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Cytophysiological studies on *Micrasterias* I. Nuclear and Cell Division

By

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(Received November 17th, 1949)

While the filamentous forms of the Conjugatae, such as *Spirogyra*, have been a classical object for numerous cytological investigations, relatively little is known about nuclear division in the unicellular Desmids, and observations *in vivo* seem to be almost lacking, though several works deal with the chromosome number found in fixed material (cp. Krieger 1937, Tischler 1942). Most of the contributions concerning nuclear division in Desmids refer to the genus *Closterium* which was the subject of a careful investigation by van Wisselingh (1913), according to whom but little could be seen in the living state in the species investigated. Czurda (1937, p. 45, 78) states that no details of nuclear division can be distinguished in the living cells of Conjugatae, even in such favourable objects as species of *Spirogyra*, while other authors report observations of the living cells of these algae (Strasburger 1880, p. 176—177; van Wisselingh 1920; Conard 1939 and other works, cp. Tischler 1942, p. 283). The nucleoli of Desmids are described in a detailed study by Kopetzky-Rechtperg (1932). Rosenberg (1940) states that there is a correspondence between the nucleolar behaviour in the living state and the division stages in *Micrasterias americana*, but he does not deal with any details of the matter in question.

Since the year 1942, the present author has made observations on the behaviour of the nucleoli during the individual development of *Micrasterias* species and, without any knowledge of Rosenberg's work,¹ has found its

¹ Owing to the war, Rosenberg's work was not obtainable in Finland before the year 1945.

connexion with nuclear division in *M. Thomasiana*, *M. rotata*, *M. radiata*, and *M. angulosa*. Moreover, it proved possible to observe the chromosomes themselves in the living state.

For various reasons the nuclear behaviour in the living cells of unicellular plants is worthy of special attention. With regard to the particular sensitivity of the cells to external agents in the state of nuclear division it might be expected that polyploid forms would arise on appropriate treatment at a suitable state. Thus Kallio (1949) succeeded in producing polyploid forms of some species of *Micrasterias*. Moreover, certain problems concerning the function of nucleus and cytoplasm can hardly be solved without the investigation of single cells at controlled stages.

Methods

In vivo observation. The cells were observed on watch-glasses with Zeiss' water-immersion objective 40 \times and oculars 7 \times and 15 \times .

The exudation of mucilage through the pores, causing movement of the cells, may hinder observation and it is therefore convenient to remove most of the mucilage previously with fine glass needles.

Cultivating. Information about the culture of these algae is given by Pringsheim (1930, 1946) and the author (Warén 1926, 1933; Waris 1939). In the present work the following solution was used:

In 10 litre solution, KNO_3 1 g, $(\text{NH}_4)_2\text{HPO}_4$ 0.2 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, CaSO_4 0.5 g, ferric citrate 0.01 g, citric acid 0.01 g, H_2SO_4 or HCl 70–80 ml of a 0.01 normal solution, pH 6.0–6.3, measured by means of the Beckman glass-electrode pH-meter. The citric acid is added in order to keep the iron in solution for a longer time (cf. Rodhe 1948, p. 35). This solution is good for *M. Thomasiana*, *M. radiata*, and *M. angulosa*, while *M. rotata* grew best in the water of the natural habitat.

If the cells are illuminated at night, the divisions will take place in the day-time, whether sooner or later depending on the physical conditions and the species in question. The time of division can be regulated by the illumination period. The temperature must not rise much above 20° C (cp. Warén 1926).

For the present work two sorts of illumination apparatus were used. One corresponds to that described by Pringsheim (1946, fig. 6), but has a cooling device of two glass globes with distilled water between them. Some copper salt was added to the water. Another is a 48 watt tubular fluorescent lamp which requires no cooling device, provided the room is not too warm. With this lamp the algae received about 80–100 foot-candles.

Fixation and staining. Fixation has been most successful with Fleming's solution and with 2BD (Darlington and La Cour 1947). After fixation with Fleming's solution, the cell-halves will readily separate, which can be in some degree prevented by adding an equal amount of about 20 % formalin solution one to several minutes after putting in Fleming's solution. After fixation, the cells must be bleached with hydrogen peroxide (3 %) before staining. Among many dyes Mayer's alum-haematoxylin according to Carazzi (cp. Schneider 1922, p. 108) has proved

most suitable. To obtain good results with it, the cells must first be overstained by keeping them about 24 hours in the solution, and then de-stained with an alcoholic solution of picric acid, diluted with water, for several hours to one day (to be controlled), washed, and cautiously transferred through the alcohol series and xylol to canada-balsam or, if so desired, to paraffin for microtome sectioning.

Centrifuging. Kallio (1949) used centrifuging to bring about polyploidy in *Micrasterias*. The cell was kept in the right position by means of folded pieces of paper embedded in agar or nutrient solution. The present author has since improved the method by using glass tubes with thin, flat, and sharp ends which makes it possible for the disciform cells to be maintained in the correct position without being damaged by the treatment. The cell is transferred by a capillary pipette into the glass tube containing nutrient solution and allowed to fall to the bottom in the right position controlled by watching with a hand lens. For the present work, centrifuging was used to displace the spindle from its proximity to the chloroplast. Centrifuging tubes with long, fine ends were formerly used by van Wisselingh (1909) for *Spirogyra*.

The general structure of full-grown cell

Micrasterias belongs, as we know, to the Desmids with a narrow median constriction, the isthmus, which joins the two symmetrical semicells (West 1905, p. 76). This structure and the size make it suitable for special observations and experiments. In the species investigated, each semicell is split into five main lobes, one polar lobe and four lateral lobes which may be called here the upper and lower side lobes (the latter bordering on the sinus). The side lobes are in their turn split into minor lobes, the extreme ones being emarginate or bidentate. The cell-wall is provided, especially in *M. Thomasiana*, with numerous distinct pores, the »pore-organs» of which will stain deeply with gentian violet. The »pore-organs» can be pressed out from the cell-wall and are then, if stained, like small nails. (The name »pore-organ» was first used by Lütkemüller, cp. Fritsch 1935, p. 341.)

The cell-wall contains, apart from cellulose, a gelatinous substance staining with methylene blue, saffranine, and ruthenium red, in a similar way to pectin (cp. van Wisselingh 1924). Apart from mucilage, drops of a yellowish fat-like substance are excreted through the cell-wall. This is striking especially in old cultures and often appears on the edge of the semicells, for instance at the sinus between them. Here the drops may join the edges of the cell-halves until these separate by division. Then some drops may be drawn out into thin threads before they are separated into two parts. In cells which have been prevented from dividing for a long time, the fat-like substance sometimes forms a girdle round the isthmus which makes observation of the nucleus difficult.

The nucleus lies in the middle of the cell, in the region of the isthmus, both ends, in normal cells, being embedded in the chloroplasts, which contain numerous pyrenoids. The nucleolus may be single as in *M. radiata*, or multiple as in *M. Thomasiana*, *M. rotata*, and *M. angulosa*. In the former case it has the shape of a rounded body, while in the latter it forms irregular masses of aggregated particles.

Common features in cell division

Cells ripe for division within one day are usually recognisable by their deep green colour, enlarged nucleus, the changes in the behaviour of the nucleoli, and by the chloroplasts having withdrawn their edges from the isthmus region. In the species with multiple nucleoli, *M. Thomasiana*, *M. rotata*, and *M. angulosa*, the first changes perceptible in the nucleolus are due to its being split into numerous irregular particles. When division is to be expected in the space of one or some hours, the particles appear as distinct granules. These are usually, but not always, at first very fine, forming cloudy groups: the »cloud stage». Eventually they become rounded, enlarge by dissolving and fusing, and congregate into one or more groups of globules like clusters of grapes: the »grape stage» (Fig. 1). The duration of these stages varies in the different species and also in different individuals of the same species. In fixed cells, the granules stain deeply with various dyes which makes it difficult for the chromosomes to be observed. It could be stated, however, in *M. Thomasiana*, that the »grape stage» corresponds to the prophase, at least the late one.

The »grape stage», and accordingly the prophase, ends strikingly with a sudden mobilisation of the granules, a moment which can be determined, in suitable cases, with the precision of some seconds. This phenomenon may be called »nucleolar mobilisation». Before this, the nucleus assumes a milky opalescent shimmer which is evidently due to increasing light refraction. This may depend on the dissolved nucleolar substance and on the orientation of the nuclear plasm into the spindle. Consequently the nucleolar granules will be partially pushed towards the edge of the spindle, where they disappear if they have not already done so within the spindle. Before disappearing, smaller nucleolar particles may fuse into larger ones. Soon after the mobilisation, from about half a minute to two minutes after its onset in *M. Thomasiana*, the nucleo-cytoplasmic surface suddenly, in a matter of seconds, breaks down, a phenomenon which is clearest in a defective form of *M. Thomasiana*. At the same time the nuclear plasm becomes cloudy.

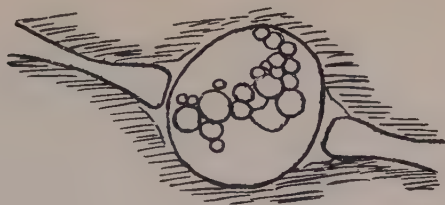


Fig. 1. *M. Thomasiana*. The nucleus at grape stage about $26 \times 22 \mu$.

Some minutes later a slight lengthwise striation may be distinguished in the spindle of the living cell.

The mobilisation of the nucleolar particles is a very marked phenomenon in the nuclear development and evidently signifies the beginning of spindle formation and accordingly that of metaphase, the beginning of which is also called metakinesis. From the fact that the mobilisation begins before the nucleo-cytoplasmic surface has disappeared it can be concluded that, in *Micrasterias*, the spindle arises inside the nucleus through lengthwise orientation of the molecular chains.

According to the description by van Wisselingh (1913, p. 417—418), the nucleolar behaviour in *Closterium Ehrenbergii* is like that in the *Micrasterias* species with multiple nucleoli.

In the species with a single nucleolus, *M. radiata*, this gradually disappears, but also in this case a movement may sometimes be observed beforehand.¹

The distribution and movements of chromosomes at metakinesis can be directly observed in *M. rotata*. The metaphase plate can be seen in all four species investigated, most clearly in *M. rotata* and *M. radiata*, while in *M. Thomasiana* it is sometimes indistinct. The chromosomes appear first as a broken row of small dots, then as an opaque transverse line in a front-view of the cell. Later they form a row of coherent double-dots indicating the approaching anaphase. Eventually two parallel rows separate signifying the beginning of the anaphase movement. This usually takes place about 15 to 20 minutes from the nucleolar mobilisation (at room temperature). The anaphase movement could be followed in *M. rotata* and *M. radiata*. After the beginning of the anaphase movement, a lengthwise striation can be distinguished in the middle of the spindle. Thus such a striation appears both before and after full metaphase, being clearer in the latter case.

The beginning of telophase is difficult to define. During the time the daughter-chromosomes are visible they appear as rows of dots. The places

¹ Kopetzky-Rechtperg (1932, p. 694) mentions *M. radiata* among the species with multiple nucleoli.

where the daughter-nuclei will appear are first dimly visible, about half-an-hour from the nucleolar mobilisation, as pale spots in the invaginations of the chloroplasts, at the same time as the new septum is initiated. Some minutes later the nucleoli begin to take shape. The nucleo-cytoplasmic surface becomes distinct only gradually, in contrast to its sudden disappearance at the end of prophase. The boundaries of the daughter-nuclei are clear before the new semicells have bulged out. The distinct boundaries signify the end of telophase.

The new septum arises, as we know, in the form of an annular ingrowth in the isthmus being initiated about half-an-hour from the nucleolar mobilisation and completed about 10—15 minutes later. During this time, often even earlier, small crystals are visible moving in the region where the septum arises. It could be observed that they come from the part of cell occupied by the chloroplast and, after the septum is complete, partially wander back. The passive movements of the crystals apparently show the direction of currents in the protoplasm, maybe in the enchylema between the fibrils (cp. Monné 1948, p. 40). It is remarkable that these currents transfer particles from distant parts of the cell-lobes towards the equator during septum formation and in the opposite direction after the septum is complete. Not all crystals behave in the same way. Some of them are visible in the region of the nucleus, probably outside this, at even earlier stages of division, and many of them still exist in the young semicells.

After septum formation is completed, the daughter-cells begin to separate causing at first a slight constriction in the isthmus. The new semicells arise as rounded bulges which become divided into first three and then five minor bulges, corresponding to the five main lobes of the adult cell. These in their turn successively divide into minor lobes until the daughter-cells are full-grown, which is possible within six to eight hours.

When the semicells begin to bulge out, the daughter-nuclei pass into them and come to lie in the cytoplasm near the isthmus. When the chloroplast later extends from the old to the new semicell, the nucleus is concealed by it and is only dimly visible. Accordingly, in the young daughter-cell the chloroplast appears as a single body being later, in the full-grown cell, divided at the isthmus, after which the nucleus is clearly visible again. Thus Czurda's interpretation (1937, p. 78) of that part of the process concerning the appearance of the daughter-nuclei in the daughter-cells, and his objection to Krieger's description (1933), is not quite correct.

The details of the division process vary in the different species and will be described below.

Micrasterias Thomasiana

The cultivated species was determined by Dr. Rolf Grönblad as *Micrasterias Thomasiana* Archer (forma ad *M. denticulata* v. *notata* Nordst. accedens, cfr. West, Monogr. II, pl. LI f. 7). It has been kept in culture since the year 1936 (cp. Waris 1939, p. 4).

Under suitable conditions, the cultivated cells reach about 220—240 μ in length and 200—220 μ in width. The isthmus is about 26 μ in diameter.

The approaching division is normally indicated by the elongated form of the nucleus which may be about 31 μ long and 22 μ broad. In this species the changes in nucleolar behaviour are especially striking and their connexion with the division stages was therefore first detected in it. The »grape stage«, in fact, derives its name from the appearance of the nucleoli during prophase in this species. The number and size of the granules vary from cell to cell, the large sizes corresponding to a relatively small number, and *vice versa*. The largest granules so far measured reached about 9—13 μ in diameter. The duration of the »grape stage« may be about $\frac{1}{2}$ —4, usually 1—2 hours. During this time the isthmus usually becomes elongated to about 9 μ making it easier to observe the nucleus.

When the nucleolar mobilisation begins, the small granules move first, the larger ones some seconds later. From the beginning of the mobilisation two to eight minutes may elapse before the last granules have disappeared. About four to six minutes from the mobilisation a lengthwise striation may be dimly visible and after six to ten minutes the chromosomes begin to arrange themselves into the metaphase plate. The splitting of the metaphase plate into two rows is often indistinct and the beginning of anaphase movement can then merely be concluded from its disappearance. This may take place about 6—8 minutes after the metaphase plate is initiated and about 15—20 minutes from the nucleolar mobilisation. It is difficult to follow the movements of chromosomes any longer.

The chromosomes were examined in fixed and stained preparations of intact cells with the exception of one paraffin section. Owing to the small size of most of the chromosomes and their appearing in groups in a front-view of the cell it was difficult to state their number exactly. In the paraffin section 34 chromosomes were counted, while in the intact cells their number was estimated at about 37. Thus, for the present, 34—37 may be regarded as the chromosome number of *M. Thomasiana*. Two of the chromosomes differ from all the others in their relatively great length, about 3—4 μ at the beginning of metaphase, while the others do not measure more than about half of this at most. One of the long chromosomes shows in a side

view a marked »knee» and possesses a very small terminal trabant at the end of an extremely thin constriction. The other long chromosomes seems to have a lateral trabant which in one preparation was doubled presumably owing to the chromosome being split into two chromatids at metaphase. The trabants could be distinguished only in some preparations. Both the long chromosomes show a slight constricted unstained part near one end. Thus they may be called »SAT-chromosomes» according to Heitz (1931) and it is possible that they correspond to the two nucleoli appearing in the young daughter-nuclei. In certain cases, however, at the earliest stages when the nucleoli became visible, they were three in number, but the possibility is not excluded that the original number was two. Apart from these long chromosomes, there is among the others one which shows a relatively long constriction, so that the joined parts may readily be taken for two different chromosomes.

In this species it was not possible to measure the speed of anaphase movement directly but in one case it was estimated by fixing the cell 2' 40" after the moment when the chromosomes appeared as a double row indicating the beginning of anaphase movement. In the stained preparation a distance of 4.6 μ between the chromosome rows was established corresponding to a speed of 1.7 μ per minute.

Plate 1.

Fig. 1—8. *Micrasterias Thomasiana* var. Different stages of one and the same cell.

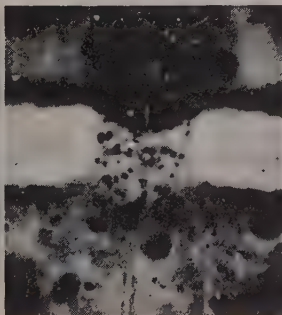
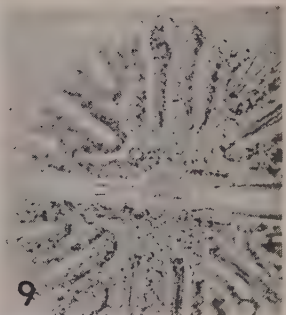
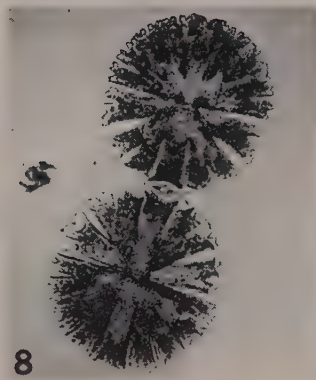
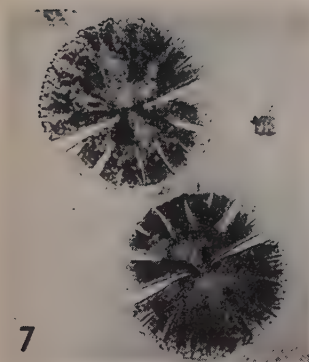
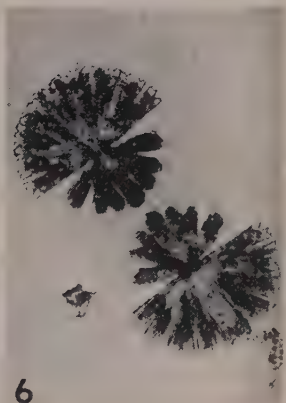
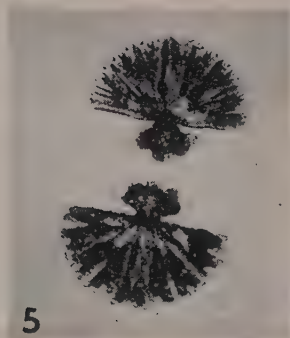
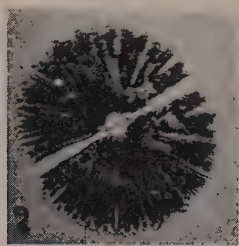
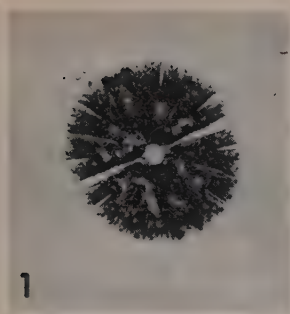
Fig. 2 ca. 116 \times , the others ca. 105 \times .

1. 11,27 a.m. Cell at early grape stage (prophase).
2. 0,41 p.m. Cell at grape stage (prophase). The transverse line in the middle is the suture of the semicells (not a septum). One of the nucleolar globules slightly visible.
3. 2,02 p.m. One hour from nucleolar mobilisation. The septum is complete, slight constriction in the isthmus.
4. 2,35 p.m. The daughter-cells begin to separate as rounded bulges.
5. 4,21 p.m. The new semicells five-lobed.
6. 5,26 p.m. The side lobes divide further.
7. 6,22 p.m. The ultimate lobes developed.
8. 8,05 p.m. The daughter-cells full-grown.

Fig. 9. *M. Thomasiana* var. Metaphase. Fixed and stained. Ca. 255 \times .

Fig. 10. *M. rotata*. Anaphase. At this stage the chromatids of the long chromosomes were still in contact with one another. Fixed and stained. Ca. 570 \times .

Fig. 11. *M. angulosa*. The beginning of metaphase, fixed soon after the nucleolar particles having disappeared. Chromosomes of equal size appear as pairs. The chromosome number was counted in this preparation. Ca. 525 \times .



Micrasterias rotata

This species is specially suitable for observing the chromosomes in the living cells, although its chromosomes are very small and numerous (plate fig. 10). The chromosome number was estimated in a paraffin section in which the nucleus was sectioned into two parts. At least 175 chromosomes were counted but their number is evidently greater. For the present, about 200 may be assumed as the chromosome number of *M. rotata*. If that proves true, this species shows perhaps the greatest haploid number to be found in normal plant cells. Among the chromosomes there are two relatively long and thread-like ones, while all the others are very small and grain-shaped. The long chromosomes may serve as characteristic if doubling is caused.

The cells may reach about 250—270 μ in length and 215—250 μ in width. At the resting stage the nucleus is broader than it is long, or as broad as it is long, barrel-shaped, about $26 \times 26 \mu$. The nucleolus is multiple, forming one or a few irregular masses. The approaching division is indicated by a slight constriction in the nucleus, the diameter of which may be about 26 μ in the middle and about 30 μ at the ends, and by the nucleolus resolving itself into a group of fine granules: the »cloud stage». This may last one to four hours, usually one and a half hours. Eventually the granules begin to fuse into larger ones as in the »grape stage» of *M. Thomasiana*, but the duration of a clear »grape stage» is much shorter in *M. rotata*, usually only one to ten minutes, although fusing of the granules may sometimes be observed for one hour.

At the beginning of the »cloud stage» the cell-halves lie in contact with one another, and at the time of the nucleolar mobilisation they are only a little separated, the isthmus being elongated to 4—5 μ at most. After the nucleolar mobilisation, the granules disappear within some minutes and the chromosomes are distinguishable as small dots evenly distributed throughout the spindle. They move towards the equator, approaching it from both poles. Before reaching it, they show in a front-view of the cell two irregular rows which can be misinterpreted as the beginning of anaphase. About 8—10 minutes from the nucleolar mobilisation, the metaphase plate begins to take shape being complete within 3—5 minutes. The anaphase begins — if the cell has not been disturbed for instance by centrifuging — about 17—22 minutes after the nucleolar mobilisation. Just before this the metaphase plate is very distinct, the part of the spindle nearest it having become cleared owing to the increased light refraction. The anaphase movement can be directly followed until the daughter-chromosomes have reached the edges of the chloroplasts, the distance between the bow-formed groups being

then about 22—26 μ , measured from the middle of the bows. It is difficult to follow the process any longer, unless the spindle has been displaced by centrifuging at metaphase. It seems that the real anaphase movement, i.e. the movement of the chromosomes through the spindle (cp. Darlington 1937, p. 29), has come to an end when the above mentioned distance has been reached. If the spindle has been displaced by centrifuging at metaphase, the rows of daughter-chromosomes may still be observed after the anaphase movement has come to an end (Plate, fig. 10).

Two cases of the division process are described below, in one of which (b) the cell was centrifuged at metaphase, in another (a) not. In (a) the nucleolar mobilisation was determined exactly, in (b) the chromosomes were already moving when the beginning of metaphase was observed.

a) Not centrifuged. The times are measured from the nucleolar mobilisation.

- 3' The granules have disappeared, chromosomes visible.
- 10' Metaphase plate is initiated.
- 14' 30" » » » complete.
- 21' 30" Anaphase begins.
- 34' 50" Distance between the chromosome groups 26.4 μ . The movement could not be followed further.
- 61' The daughter-nuclei and nucleoli begin to be distinguishable.
- 79' Daughter-nuclei still more distinct. In one of them two large nucleoli visible. The new septum begins to split by a slight constriction in the isthmus.
- 90' The daughter-nuclei are oblong, about $22 \times 13 \mu$.

b) Cell centrifuged at metaphase. Time in hours, minutes, and seconds.

- 1,25,00 p.m. The cell was found at the »cloud stage» with signs of fusion of the granules.
- 1,50,00 » Metakinesis. Nucleolar mobilisation is over.
- 1,57,30 » Metaphase plate is complete.
- 2,16,20 » Anaphase begins.
- 2,24,15 » Distance between the chromosome groups 17.6 μ .
- 2,25,10 » The places where the daughter-nuclei will arise dimly visible.
- 2,30,30 » Distance between the chromosome groups 22 μ .
- 2,31,15 » The rows of chromosomes are shortened, less distinct.
- 2,32,05 » Distance 24 μ .
- 2,32,50 » The new septum is initiated.
- 2,33,50 » The chromosome rows seem to disperse.
- 2,34,40 » Dots of the chromosome rows still distinguishable.
- 2,35,50 » Of the chromosome rows nothing can be distinguished. The new septum on both sides as an ingrowth of 6.5 μ .
- 2,42,00 » The sites of daughter-nuclei appear as pale spots without distinct boundaries.
- 2,46,00 » Septum nearly complete. One of the daughter-nuclei has reached the edge of the chloroplast.
- 2,50,00 » Nucleoli dimly visible.
- 2,57,00 » Nucleoli distinct.

- 2,59,00 p.m. The boundary of one nucleus relatively distinct, that of another not.
 3,25,00 » Both daughter-nuclei distinct, in one of them two nucleoli distinguishable.
 3,57,00 » The new semicells as bulges of about 35 μ width and 18 μ length.

The following data show the speed of anaphase movement in the cells described above (unit of the eyepiece scale=4.4 μ):

Distance μ	Time past from the beginning	
	a	b
4.4	2'	1' 5"
8.8	3' 50"	4' 25"
13.2	6' 25"	6' 30"
17.6	8' 10"	7' 55"
20.0	—	11'
22.0	11' 10"	14' 10"
24.0	—	15' 45"
26.4	13' 20"	—

For both cells the times at the distance of 13.2 and 17.6 μ correspond well with one another and from them an average speed of about 2 μ per minute can be reckoned.

Micrasterias angulosa

The cultivated cells reach the size of about 240×190 to 250×200 μ . The nucleus may be 26×26 to 31×26 μ . The nucleolus is multiple showing at prophase a finely granulated »grape stage«. At this stage the isthmus is only a little elongated, about 4 μ .

The fine-granular stage may last about $1 \frac{1}{2}$ —2 hours. Two to five minutes after the nucleolar mobilisation small dots are visible distributed throughout the spindle, taking later, about ten minutes from the mobilisation, a worm-like shape, the length of the particles being probably under 0.5 μ . They were considered as chromosomes. The metaphase plate begins to take shape about 12—13 minutes from the nucleolar mobilisation being completed in two minutes. About eight minutes it was visible as an opaque transverse line in the spindle, the last three minutes as a double row of dots. The anaphase movement was observed to begin about 22 minutes from the nucleolar mobilisation and came to an end about 10 minutes later, the distance between the chromosome rows being then about 22 μ corresponding to a speed of about 2 μ per minute. In this species, however, it was very difficult to follow the anaphase movement *in vivo*. About 45 minutes from the nucleolar mobilisation the new septum was nearly complete and after 50 minutes

the boundaries of the daughter-nuclei were distinct indicating the end of telophase. Thus the duration of mitosis can be estimated as follows: Prophase $1\frac{1}{2}$ —2 hours, whole metaphase about 20 minutes, anaphase about 10 minutes, telophase about 20 minutes.

The chromosome number was counted in a fixed and stained preparation by three observers and was found to be 60—64, or about 60. All the chromosomes are very small and granular (plate, fig. 11). Many of them appear as pairs of two equal grains.

Micrasterias radiata

The cells observed were 92×88 to $158 \times 158 \mu$ in size. The nucleus is round or oblong, about 18μ in diameter. It shows, in full-grown cells, a single rounded nucleolus of about 9μ in diameter.

The approaching division is indicated by the nucleolus showing an uneven, granular surface. Then the nucleolus gradually disappears without any sharp end-point. Sometimes, however, a movement may be observed just before it disappears. Metaphase and the beginning of anaphase can be clearly observed.

Example. The times have been measured from the disappearance of the nucleolus.

- 6' 50" The metaphase plate begins to take shape.
- 9' " " " shows a row of double dots.
- 11' " " " is flattened, i.e. the row is narrower.
- 20' 20" Anaphase begins. A double row of dots is visible.
- 21' 30" Distance between the rows 5.3μ .
- 24' The rows have reached the edges of the chloroplasts, being concealed by them.
- 25' The new septum is initiated. Crystals visible in the region of it.
- 30' 30" The isthmus is about 5μ long.
- 40' The initial septum goes across the whole isthmus.
- 41' The crystals have removed from the middle.
- 50' The sites of daughter-nuclei dimly visible as pale spots.
- 53' The new septum is complete.
- 56' First signs of nucleoli.
- 61' Daughter-nuclei clearer. Constriction of the isthmus begins.
- 67' Daughter-nuclei distinct with granules on the septum side. In each of them two nucleoli, a large and a small one symmetrically placed.
- 92' The new semicells begin to bulge out.
- 107' Daughter-nuclei about 16μ in diameter. The large nucleolus about 6μ , the small one 2μ in diameter.
- 168' The new semicells begin to form lobes.
- 171' The new semicells about 37μ broad and 26μ long, daughter-nuclei about $13 \times 18 \mu$.

It is remarkable that in the young daughter-nuclei two nucleoli occurred. In another case, only in one of the daughter-nuclei were two nucleoli observed, the smaller of which later became indistinct.

In a cell fixed 1' 30" from the beginning of anaphase, the distance between the chromosome groups was 12 μ , corresponding to a speed of 8 μ a minute, which may be too high a value.

According to the observation *in vivo* described above, the anaphase movement may have a speed of 4.6 μ a minute.

The chromosomes are very small and numerous. Their number could not be estimated.

Summary

The *in vivo* observation of nuclear division is possible in *Micrasterias rotata*, *M. Thomasiana*, *M. angulosa*, and *M. radiata*, in the different species to a varying extent.

In the three first-named species with a multiple nucleolus, the approaching division is indicated by the nucleolus resolving itself into groups of distinct granules which are usually first very fine and somewhat irregular, the »cloud stage», later owing to dissolving and fusing larger and globular, the groups then resembling clusters of grapes, the »grape stage». The granular stage corresponds to the prophase, the »grape stage» representing at least the later part of it. The »grape stage» may last in *M. Thomasiana* from half an hour to four hours, in *M. rotata* from half a minute to ten minutes. It ends with a sudden mobilisation of the granules, the »nucleolar mobilisation», which signifies the beginning of spindle formation and can be determined, in suitable cases, with a precision of some seconds.

In *M. radiata*, which possesses a single nucleolus, the approaching division is indicated by the edge of the nucleolus becoming uneven, as if granulated, before the nucleolus gradually disappears. Also in this case a movement of the nucleolus may sometimes be observed.

The chromosome number is in *M. Thomasiana* about 34—38, in *M. angulosa* 60—64, in *M. rotata* about 200. In *M. Thomasiana* and *M. rotata* two of the chromosomes are especially conspicuous owing to their length. In *M. Thomasiana* both the long chromosomes show an unstained portion near one end and possess a trabant.

The original number of nucleoli appearing in the young daughter-nuclei in *M. Thomasiana* and *M. rotata* is often two. Even in *M. radiata* two nucleoli, a large and a small one, may sometimes be observed in the young daughter-nuclei.

The movements of chromosomes during metaphase and anaphase can be

observed in *M. rotata*, during anaphase in *M. radiata* and less distinctly in *M. angulosa*. The metaphase plate can be distinguished in all four species investigated, less distinctly in *M. Thomasiana*. The speed of anaphase movement at room temperature was estimated in *M. rotata* and *M. angulosa* at about 2 μ per minute, in *M. radiata* at 4.6 μ per minute.

The duration of mitosis in the different species at room temperature is shown by the following data:

Species	Prophase	Metaphase			Anaphase	Telophase
		beginning	full	whole		
<i>M. Thomasiana</i>	1½–4 hours	7–18'	2–5'	12–20'	4–9'	15–25'
<i>M. rotata</i>	1 hour	10–13'	10–13'	21–24'	6–12'	30–45'
<i>M. radiata</i>	—	6–11'	4–8'	12–20'	7'	20–47'
<i>M. angulosa</i>	1½–2 hours	13–14'	8'	20'	10'	20'

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References

- Conard, A.: Sur le Mécanisme de la Division Cellulaire et sur les Bases Morphologiques de la Cytologie. — Université Libre de Bruxelles. Travaux du Jardin Expérimental Jean Massart. 1939.
- Czurda, Viktor: Conjugatae. — Linsbauer, Handbuch der Pflanzenanatomie VI: 2. Berlin 1937.
- Darlington, C. D.: Recent Advances in Cytology. — Second Edition. London 1937.
- and La Cour, L. F.: The Handling of Chromosomes. — Revised Second Edition. London 1947.
- Fritsch, F. E.: The Structure and Reproduction of the Algae. — Vol. I. Cambridge 1935.
- Heitz, E.: Die Ursache der gesetzmässigen Zahl, Lage, Form und Grösse pflanzlicher Nukleolen. — Planta 12: 775. 1931.
- Kallio, Paavo: Artificially Produced Binuclear, Diploid and Anuclear Desmids. — Arch. Soc. Zool. Bot. Fenn. »Vanamo» 2: 92. 1949.
- Kopetzky-Rechtperg, Oskar: Die Nukleolen im Kern der Desmidiaceen. — Beihefte zum Bot. Centralblatt 49: 686. 1932.
- Krieger, W.: Die Desmidiaceen. — Rabenhorst, Kryptogamen-Flora 13, 1933. — 2. Auflage 1937. — 2. Teil, Lief. 1. 1939.
- Monné, Ludwik: Functioning of the Cytoplasm. — Advances in Enzymology 8: 1. 1948.
- Pringsheim, E. G.: Die Kultur von Micrasterias und Volvox. — Abdruck aus Arch. für Protistenkunde 72: 1. 1930.
- Pure Cultures of Algae. — Cambridge 1946.
- Rodhe, Wilhelm: Environmental Requirements of Fresh-water Plankton Algae. — Symbolae Botanicae Upsalienses 10: 1. 1948.
- Rosenberg, M.: Formation and Division of Binucleate Giant Cells in *Micrasterias americana* (Ehrenb.) Ralfs. — The New Phytologist 39: 80. 1940.
- Schneider, Hans: Die botanische Mikrotechnik. — Jena 1922.
- Strasburger, Ed.: Zellbildung und Zelltheilung. — Jena 1880.

- Tischler, G.: Allgemeine Pflanzenkaryologie. 2. Kernteilung und Kernverschmelzung. Zweite Auflage. — Linsbauer, Handbuch der Pflanzenanatomie II: 1. Berlin-Zehlendorf 1942.
- Warén, Harry: Nahrungsphysiologische Versuche an *Micrasterias*. — Soc. Scient. Fenn., Comment. Biol. II, 8: 1. 1926.
- Über die Rolle des Calciums im Leben der Zelle auf Grund von Versuchen an *Micrasterias*. — *Planta* 19: 1. 1933.
- Waris, Harry: Über den Antagonismus von Wasserstoffionen und Metallkationen bei *Micrasterias*. — *Acta bot. fenn.* 24: 1. 1939.
- West, W.: A Monograph of the British Desmidiaceae. Vol. II. London 1905.
- van Wisselingh, C.: Zur Physiologie der Spirogyrazelle. — Beihefte zum Bot. Centralblatt 24: 133. 1909.
- Über Kernstruktur und Kernteilung bei *Closterium*. — *Ibidem*, 29: 409. 1913.
- Über Variabilität und Erbllichkeit. — *Zeitschrift für induktive Abstammungs- und Vererbungslehre* 22: 65. 1920.
- Die Zellmembran. — Linsbauer, Handbuch der Pflanzenanatomie III: 2. 1924.

The Effect of Glutathione on the Growth-Inhibiting Substances in Resting Potato Tubers

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The occurrence of large quantities of growth-inhibiting substances in the peelings of resting potatoes and the fact that these substances have, to a great extent, disappeared at the termination of the rest-period, has earlier been demonstrated (Hemberg 1946 and 1947). It has, consequently, been assumed that the growth-inhibiting substances constitute at least one of the causes of this rest. Later (Hemberg 1949 a), it has been ascertained that when resting potatoes are treated with ethylenechlorhydrin, which has long been known to have a rest-breaking effect, the growth-inhibiting substances begin to disappear as soon as four days after the treatment has been initiated.

Guthrie (1933) found that the glutathione content is increased in resting potatoes treated with ethylenechlorhydrin. He assumed that the rest-breaking effect of ethylenechlorhydrin is due to its capacity to cause an increase in the glutathione content of the potato. As a matter of fact, Guthrie (1940) succeeded in breaking the rest of potatoes by direct treatment with glutathione. However, he was unable to observe any increase in the glutathione content in potatoes interrupting their rest by natural means.

Investigations by Emilsson (1949) have shown that the glutathione content of potatoes is increased towards the end of the rest-period. After that, it decreases somewhat, though never so far as the original value at the beginning of the rest-period. Emilsson concludes that the increase of glutathione towards the end of the rest-period as well as the disappearance of growth-inhibiting substances, ascertained by Hemberg towards the end of the same period, are of significance for the termination of the rest.

The purpose of the present investigation is to establish whether there is any connection between the glutathione increase in the potato towards the end of the rest-period and the disappearance of the growth-inhibiting substances.

Experimental results

The apical tops of a number of potatoes of the Magnum bonum variety were cut off so as to obtain top pieces of about 5 gm, respectively. These tops were washed for three hours in running water in order to remove the content of wounded cells. The pieces were then placed in petri dishes containing a 2 % glutathione solution, or distilled water. In the former case, the potato pieces will be referred to below as the glutathione-treated ones, in the latter, as the water-treated ones. The dishes were kept in a box with moist air at 20° C. In order to prevent any ethylene effects from coal-gas at the laboratory, fresh air was blown from without into the room where the box was kept during the whole investigation period. After 2, 3, and 4 24-hour-periods, five of the glutathione-treated potato pieces were taken out, washed in distilled water and peeled, the peelings being extracted with ether. At the same time, five of the water-treated potato pieces were given identical treatment. The impure ether extracts were evaporated to a dry state and the residue dissolved in chloroform. The extracts were analysed with regard to their content of growth-inhibiting substances, in the same manner as in previous investigations (Hemberg 1946, 1947, 1949 a and b), by determination of the capacity of the extracts to inhibit the effect of indole acetic acid in the *Avena* test.

All the peel extracts from the water-treated potato pieces showed a pronounced inhibiting effect on indole acetic acid in the *Avena* test (see Figs. 1 A, C, E, and G). Accordingly, they contained a large quantity of growth-inhibiting substances. The same applied to the peel extract from the glutathione-treated potato pieces which had been left in the glutathione solution for 2 24-hour periods (see Fig. 1 B). On the other hand, in the peel extracts from the glutathione-treated pieces which had been placed in the solution for 3 and 4 24-hour-periods, respectively, the greater part of the substances had disappeared (see Figs. 1 D, F, and H).

The experiments were not carried out under aseptic conditions. Consequently, a certain bacterial growth occurred when the potato pieces had been left in the solutions for 4 days. Still, this cannot have contributed to the disappearance of the growth-inhibiting substances, seeing that, in both the experiments that lasted for 4 days, the bacterial development was

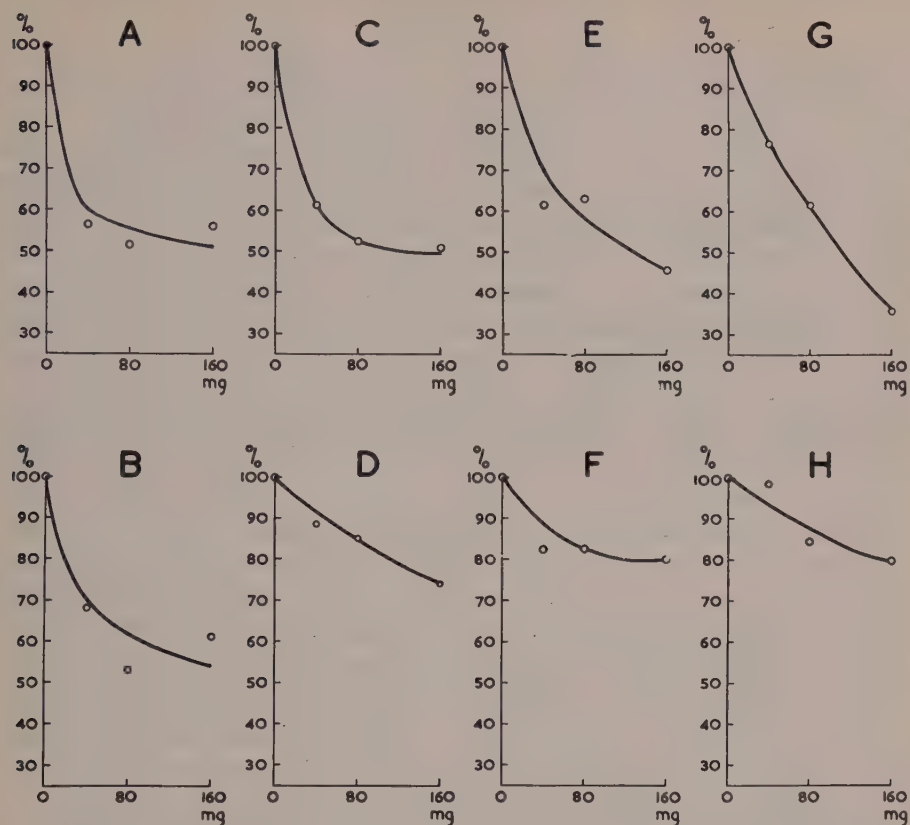


Fig. 1. The inhibition and non-inhibition of the effect of indole acetic acid in the *Avena* test by impure extracts from potato peelings from resting potatoes not treated, and treated with glutathione. Experiments with agar discs containing the same amount of indole acetic acid, but varying amounts of extracts. A, C, E, and G: extracts of peelings of pieces of potato treated with distilled water. B, D, F, and H: extracts from peelings of pieces of potato treated with 2 % glutathione 2, 3, 4, and 4 days, respectively, after the commencement of the treatment. A, E, B, and F were initiated on Oct. 6th, 1949, C, G, D, and H on Nov. 5th, 1949. For details regarding the treatment, see the text. Abscissa: mg of peelings corresponding to 0.1 ml agar. Ordinate: recovered amount of indole acetic acid in percentages of the amount in the control experiments.

much less conspicuous in the glutathione solution than in that with distilled water.

No attempts were made to determine the extent of shortening of the rest-period owing to the glutathione treatment on account of the expensiveness of the glutathione preparation employed. Instead, reference may be had to Guthrie's experiments in 1940.

Discussion

It is evident from the experiments that glutathione treatment of resting potatoes causes the disappearance of growth-inhibiting substances from the potatoes. The fact that an ethylenechlorhydrin treatment has the same effect (Hemberg 1949 a) is therefore, undoubtedly, due to the subsequent increase of the glutathione content in the potato, as stated by Guthrie (1933). The disappearance of growth-inhibiting substances towards the end of the rest-period of potatoes that have broken their rest by natural means can, thus, be attributed to the increase, observed by Emilsson (1949), in the glutathione content in the potatoes towards the termination of that period.

The interruption of the rest is, without a doubt, a very complicated process involving many different, interplaying, reactions. Since glutathione causes a disappearance of the growth-inhibiting substances, it would seem to favour one of the earlier reactions in this process. This, in turn, leads to the destruction of growth-inhibiting substances, perhaps via other reactions. When these substances have disappeared, the rest ceases and the previously inhibited sprouts can begin to grow. On the other hand, the auxins probably play no part in the resting of the potato (Hemberg 1947 and 1949 a).

The fact that the growth-inhibiting substances regulate the rest does not, apparently, concern potatoes only. Thus, it has been demonstrated (Hemberg 1949 b) that these substances are found in resting terminal buds of *Fraxinus*, but have disappeared from the terminal buds that have broken their rest. This disappearance of the growth-inhibiting substances is produced by ethylenechlorhydrin treatment also in resting *Fraxinus* buds.

Summary

A description is given of the effect of glutathione on growth-inhibiting substances in resting potatoes. Potato pieces, consisting of the apical parts of the tubers, weighing about 5 gm, respectively, were deposited, after careful washing for three hours in running water, in petri dishes containing distilled water, or a 2 % glutathione solution which, according to Guthrie (1940), breaks the rest-period. After 2, 3, and 4 24-hour-periods, 5 water-treated, and 5 glutathione-treated potato pieces were removed, washed and peeled, the peelings being extracted with ether. The extracts were analysed with regard to their content of growth-inhibiting substances by means of the *Avena* test. It was found that the peel extracts from the pieces that had been placed in a glutathione solution for 3 and 4 24-hour-periods, respectively, contained a very small quantity of growth-inhibiting substances,

while the extracts from the pieces that had only been forty-eight hours in a glutathione solution, or 2, 3, and 4 24-hour-periods, respectively, in distilled water, all without exception, contained growth-inhibiting substances in great quantities and in almost equal amounts.

Thus, it is evident that the glutathione solution causes the growth-inhibiting substances to disappear from the tissues.

The interruption of the rest-period is, in all likelihood, a complicated process involving many different reactions, where one of the earliest reactions is affected by the glutathione. This reaction then, possibly via other reactions, leads to the destruction of the growth-inhibiting substances, thus enabling the previously inhibited sprouts to begin to grow.

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References

- Emilsson, B.: Studies on the rest period and dormant period in the potato tuber. — *Acta Agr. Suec.* III:189. 1949.
- Guthrie, J. D.: Change in glutathione content of potato tubers treated with chemicals that break the rest period. — *Contrib. Boyce Thompson Inst.* 5:331. 1933.
- Role of glutathione in breaking of the rest period of buds by ethylene chlorohydrin. — *Contrib. Boyce Thompson Inst.* 11:261. 1940.
- Hemberg, T.: Wachstumshemmende und wachstumsfördernde Stoffe bei der Kartoffel. — *Arkiv f. Bot.* 33 B, No. 2. 1946.
- Studies in auxins and growth-inhibiting substances in the potato tuber and their significance with regard to its rest-period. — *Acta Hort. Berg.* 14:133. 1947.
- Significance of growth-inhibiting substances and auxins for the rest-period of the potato tuber. — *Physiol. Plant.* 2:24. 1949 a.
- Growth-inhibiting substances in terminal buds of *Fraxinus*. — *Physiol. Plant.* 2:37. 1949 b.

Studies in Physiological Analysis of Yield I. Varietal Differences in Photosynthesis in the Leaf, Stem, and Ear of Wheat

By

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Introduction

Crop yield is a net result of the various metabolic processes of the plant which are closely interlinked and the analysis of which is, therefore, beset with considerable difficulty. Attempts have nevertheless been made to investigate this intricate relationship and the pioneer work of Balls (1915 onwards) on cotton deserves special mention in this connection.

During a discussion on the choice of developmental observations for forecasting yield, Gregory (1929) pointed out that plant physiologists were not in a position to state which physiological activities were in the direct line of yield and, therefore, such observations as were arbitrarily undertaken, and without appreciation of their significance under a particular environment, for the purpose of interpreting differences in yield may not serve much useful purpose. Later on, employing Gregory's technique of growth analysis, Crowther (1934) was able to account for the interaction of nitrogen and water supply on the yield of seed cotton in the Sudan in terms of maximum leaf growth and plant height. The larger height meant increase in the number of leaves and fruit-bearing branches. Since the net assimilation rate was not affected under the conditions obtaining in the Sudan, the yield was found to be related to leaf weight. This correlation did, not, however, hold good in Egypt and Crowther (1938) explained it as due to loss of bolls in the lower branches through bollworm attack.

Watson (1947) also accounted for the seasonal differences in the weight of total dry matter of the crop in terms of leaf area in the case of barley, potato, mangold, sugarbeet and wheat.

Went (1944) observed that under controlled conditions of nutrition, water supply, duration and intensity of light and relative humidity, day temperature of 26°C and night temperature of 18°C were optimal for growth and fruit production. At night temperature above 18°C rate of sugar transport limited growth of stem as well as of roots and fruits; below 18°C rate of growth process itself became limiting. The process with an optimal temperature of 26°C , or higher, which controlled growth during the day was probably photosynthesis or salt uptake.

Plant breeders have also devoted considerable attention to the study of correlation between varietal difference in yield and various attributes like ear number and its size, grain number and its size, etc., with a view to seeing how far it is genetically determined. Their experience, in general, indicated that these yield components being quantitative in nature have a complicated mode of inheritance and are influenced more or less by environment. In such studies the technique of correlation analysis and discriminant functions is usually adopted, which because of its empirical nature cannot be expected to lead to an understanding of the relation of particular attributes of development to yield which, in fact, is the object of plant physiology.

Worzella (1941) has suggested that long and short season plants, tall and short varieties, wide and narrow leaves, rate of growth, sterility, intensity of chlorophyll etc., may be inherent factors affecting yield. He had apparently in mind differential assimilatory capacity of the varieties when he emphasized such factors as season, plant height, leaf width, chlorophyll concentration etc.,

Boonstra (1929) suggested from his observation on 4 varieties of oats that the »Length of life» of the leaves (and presumably, therefore, the duration of the assimilatory activity) was an important factor in determining yield. Boonstra (1934) also analysed the differences between root system of 7 varieties of peas in relation to differences in yield and concluded that variations in root activity, permeability etc., are varietal characters and not due to chance environmental factors.

In the investigation of correlation between physiological activities and yield, the effect of environmental conditions during the fruiting stage deserves special consideration. Knowles and Watkin (1931) and Watson and Norman (1939) have observed in the case of wheat and barley, respectively, that about $\frac{1}{3}$ of the final dry weight of the plant is added during the post-ear emergence period. In view of this observation the assimilation by the plant during this period would seem to merit special consideration as a factor in determining final yield. It may also be noted that after ear emergence the

proportion of green leaf surface to that of other green parts diminishes appreciably with time and the assimilation by the latter might be expected to contribute substantially to ultimate dry matter output. In fact, Boonstra (1931), Smith (1933), Watson and Norman (1939) and Archbold (1942) found that, in cereals, assimilation by stems and ears contributed substantially to grain yield.

As a preliminary step in the physiological analysis of factors determining yield we have, therefore, investigated possible varietal differences in respect of the contribution made by assimilation in the leaves, stems and ears to grain yield during the post-ear emergence period. The experiment was first carried out during the 1947—48 season and a report on these observations was recently published by us (Asana and Mani, 1949). It was shown that contributions by photosynthesis in the ear and the leaf differed substantially among the five varieties studied. Encouraged by this observation, a more detailed investigation was undertaken during the 1948—49 season and an account of this is presented here.

Experimental Procedure

The technique adopted was more or less similar to that of Watson and Norman (1939). The contribution of the assimilation by different organs was estimated indirectly by severing the leaves or shading the ear. Thus the treatments comprised control, defoliation (removal of green leaf blades after ear emergence), shading the ear and a combination of the last two. The fourth treatment enabled only the stem with leaf sheaths to assimilate.

All the four treatments were included in each pot, there being two shoots per treatment. There were ten such pots for each variety. In the 1947—48 experiment the initial sample was taken soon after dehiscence was noticed and the treatments also commenced simultaneously. Since all the shoots did not dehisce on the same day, the treatments and the initial samples were distributed among the shoots in such a way that the mean number of days from sowing to the time at which treatment commenced was approximately equal in all cases. In 1947—48 only 8 comparable shoots (as regards ear size, plant height and number of spikelets per ear) were available per pot and, therefore, initial samples were taken from 8 other pots. In the 1948—49 experiment the following changes were introduced: —

(a) 10 plants were grown per pot, 8 for four treatments and 2 for the initial sample; (b) only mother shoots were taken and the tillers were cut off immediately after the treatments commenced; (c) a shoot was either treated or taken for initial sample only on the sixth day after anthesis and for this

purpose the date on which every ear dehisced was carefully noted. The idea behind commencing the treatment on the sixth day after dehiscence was to ensure that fertilization and embryo development were not in any way affected adversely by the treatments, it having been established that embryo formation is complete within six days.

The ear was shaded by enclosing it in a light-proof double-walled cylinder made of cartridge paper. Small holes pierced in the walls of the cylinder permitted free circulation of air. Ordinary unglazed garden pots were used, each pot having a capacity to hold about 22 lbs. of air-dry soil.

Calculation of respective contributions of different organs to net assimilation and grain weight

The 1947—48 data were worked out on the lines suggested by Watson and Norman (1939). These workers did not weigh the grain and the chaff separately and estimated the contribution by photosynthesis in the ear to grain weight on the assumption the grain formed about 85 % of the ear weight. In view of the fact that the chaff also gained in weight after dehiscence, a slightly modified method was used for computing the 1948—49 data and is fully illustrated below.

Treatment ¹	Initial dry weight per shoot in gm				Final dry weight per shoot in gm				Gain in total dry weight or net assimilation per shoot in gm			
	Leaf	Stem	Ear	Total	Leaf	Stem	Ear	Total	Leaf	Stem	Ear	Total
C	0.29	1.23	0.39	1.91	0.29	1.18	1.70	3.17	0.00	— 0.05	1.31	1.26
D	—	1.23	0.39	1.62	—	1.14	1.25	2.39	—	0.09	0.86	0.77
S	0.29	1.23	0.39	1.91	0.31	1.18	1.55	3.04	0.02	0.05	1.16	1.13
D + S	—	1.23	0.39	1.62	—	1.10	0.87	1.97	—	0.13	0.48	0.35

¹ C for control, D for defoliation, S for ear shading.

The mean effect on net assimilation of defoliation and ear shading works out as:

	Control	Defoliation	Mean effect of	
			Defoliation	Shading
Shading	1.26	0.77	2.03	
	1.13	0.35	1.48	
	2.39	1.12		
			$(2.39 - 1.12)/2$	$(2.03 - 1.48)/2$
			= 0.635	= 0.275

Thus to the gain in total weight of 1.26 gm the respective contributions by photosynthesis in the ear, leaf and stem were 0.275 gm. 0.635 gm and 0.350 gm. The leaf, therefore, contributed 50 %, the ear 22 and the stem 28 %.

It will be seen that the whole of the dry matter assimilated during this period accumulated in the ear.

The grain and the chaff in the initial ear sample amounted to 0.10 and 0.29 gm. and in the final sample to 1.34 and 0.36 gm respectively. Thus the chaff gained in weight by 0.07 gm. and the grains by 1.24 gm. The chaff also gained in weight under the different treatments as shown below:

	Control		Defoliation	
	0.07		0.02	
	0.01		0.00	
	0.08		0.02	

Thus the leaf contributed 0.03 gm to increase in chaff weight and the ear 0.04. The contributions to grain weight by leaf and ear were, therefore, corrected accordingly and the increase in grain weight of 1.24 gm, after anthesis, was accounted for as shown below:

Due to	Net assimilation		Grain weight	
	Absolute	Percent	Absolute	Percent
Leaf	0.635 gm	50	0.605 gm	49
Ear	0.275 gm	22	0.235 gm	19
Stem	0.350 gm	28	0.350 gm	28
Reserve	—	—	0.050 gm	4
	1.260	100	1.240	100

Since the stem lost weight by 0.05 gm at the end of the period, this amount which was added to the grain cannot be regarded as a contribution by photosynthesis in the stem and is, therefore, considered as derived from reserve material.

Data on the lines indicated above were calculated for each replicate and the difference between the varieties and the treatments in respect of contributions made by photosynthesis in each organ to net assimilation (from 5 days after anthesis till harvest) and to grain weight was tested statistically by the analysis of variance.

Experimental Data

In 1947 five varieties viz. N.P.¹ 710, N.P. 165 Punjab 9D Agra Local and N.P. 735 were taken for study. The first 4 varieties are early to medium and

¹ N.P. stands for New Pusa, formerly designated P (for Pusa) and I.P. (for Imperial Pusa) respectively. These varieties have been evolved at the Indian Agricultural Research Institute. All the varieties used in this experiment are vulgare wheats and have ears with awns, except N.P. 165 which has no awns and CPH 47, which has very short awns.

the last named late. N.P. 735 being late, does not do well under Delhi conditions due to the prevalence of hot and dry weather during the period of grain formation. In 1948 N.P. 735 was, therefore, omitted and two more varieties viz., N.P. 52 and CPH 47, were included. The mean percentage contribution by assimilation in the leaf, ear and stem to net assimilate is indicated in table 1.

Table 1.

Variety	Percentage contribution by photosynthesis in		
	Leaf	Ear	Stem
N.P. 165	35.5	23.7	40.8
Agra Local.....	37.8	34.6	27.5
N.P. 52	28.8	30.2	41.0
N.P. 710	23.4	45.9	30.7
CPH 47	44.9	18.4	36.7
Punjab 9 D	26.0	29.8	44.2

Critical difference at 5 % probability, 17.7.

Critical difference at 10 % probability, 14.8.

Significance of differences has been judged throughout at 10 % level of probability because some of the differences miss significance at 5 % level by a very narrow margin.

It will be seen that the percentage contribution by the ear in N.P. 710 was the highest; it was, however, significantly superior to that in N.P. 52, Punjab 9D, N.P. 165 and CPH 47 only. The contribution by the ear in Agra Local was significantly superior to that in CPH 47 only. Among all the three organs photosynthesis in the ear contributed the most to net assimilate in N.P. 710. In view of the observations of Miller, Gauch and Gries (1944) and Vervelde (1946) that awns on the ears enhance the photosynthetic activity of the latter, the lowest contribution by the ear to net assimilation in N.P. 165 and CPH 47 may be accounted for. Considering that the contribution of the ear of N.P. 710 was, however, superior to that of N.P. 52 and Punjab 9D, all of which have awns, this explanation would appear to lose some of its force.

The percentage contribution by photosynthesis in the leaf was the highest in CPH 47, although it was significantly superior to that in N.P. 52, Punjab 9D and N.P. 710 only. The contribution in Agra Local was superior, although not significantly, to that in N.P. 710 only. Among the three organs photosynthesis in the leaf contributed the highest to net assimilation in CPH 47, although it was significantly higher than that of ear only.

The percentage contribution by photosynthesis in the stem was the highest in Punjab 9D, although it was significantly higher than that in Agra Local

only. Among all the three organs, photosynthesis in the stem contributed the most to net assimilate in Punjab 9D.

The mean percentage contribution by photosynthesis in the different organs and from reserve material to grain yield is shown in table 2.

Table 2.

Variety	Contribution by			
	Leaf	Ear	Stem	Reserve
N.P. 165	34.2	16.9	42.8	6.1
N.P. 710	20.1	35.2	22.3	22.1
Agra Local	34.5	28.2	26.0	11.3
Punjab 9D	23.5	26.6	43.9	6.0
N.P. 52	28.7	27.3	37.0	7.0
CPH 47	42.2	15.5	35.6	6.7

Critical difference at 5 % probability 13.2.

Critical difference at 10 % probability 11.1.

The percentage contribution by the ear varied as indicated below:

N.P. 710	Agra Local	N.P. 52	Punjab 9D	N.P. 165	CPH 47
35.2	28.2	27.3	26.6	16.9	15.5

The contribution by photosynthesis in the ear to grain yield was the highest in N.P. 710 and the varieties well in the same order as in the case of contribution to net assimilate. The contribution in N.P. 710 and Agra Local was, however, significantly superior to that in CPH 47 and N.P. 165, and in N.P. 52 and Punjab 9D to that in CPH 47 only.

The percentage contribution by the leaf varied as:

CPH 47	Agra Local	N.P. 165	N.P. 52	Punjab 9D	N.P. 710
42.2	34.5	34.2	28.7	23.5	20.1

The contribution by photosynthesis in the leaf to grain yield was the highest in CPH 47, although it was significantly superior to that in N.P. 710, Punjab 9D and N.P. 52 only. The contribution by the leaf to grain yield in Agra Local and N.P. 165 was also superior to that in N.P. 710. The general order of varieties was more or less the same as in the case of contribution by the leaf to net assimilate.

The percentage contribution by the stem varied as:

Punjab 9D	N.P. 165	N.P. 52	CPH 47	Agra Local	N.P. 710
43.9	42.8	37.0	35.6	26.0	22.3

Again the order of varieties is similar to that obtained in the case of contribution to net assimilate, but the varietal differences are wider in this

case. The contribution in N.P. 165 and Punjab 9D was significantly greater than that in Agra Local and N.P. 710, whereas that in N.P. 52 and CPH 47 was superior to that in N.P. 710 only.

The percentage contribution from the reserves varied as:

N.P. 710	Agra Local	N.P. 52	CPH 47	N.P. 165	Punjab 9D
22.4	11.3	7.0	6.7	6.1	6.0

In N.P. 710 the contribution from the reserves was the highest and was significantly greater than that in all other varieties.

These calculations indicate that the contributions of the dry matter to the grain from photosynthesis in the leaf, ear and stem differ with varieties. In view of these differences it is worth considering how these varieties would yield under such external conditions as adversely affect the photosynthetic activity of the shoot after ear emergence.

One may expect, for instance, that under such conditions a variety like N.P. 710 would perform relatively better as compared to the other varieties. Moreover, if such an expectation is realised it would lend confidence to the method of estimating these contributions, for, the grain yield of the treated plants was not taken into consideration in these calculations. To test these

Table 3.

Variety	C	D	S	D + S	
N.P. 165	1.61	1.02	1.22	0.67	Net assimilation in gm per shoot
	1.44	1.06	1.23	0.90	Grain yield in gm »
	+ 0.17	- 0.04	- 0.01	- 0.23	Difference
N.P. 710	1.58	0.93	0.74	0.39	Net assimilation in gm »
	1.65	1.28	1.16	0.80	Grain yield in gm »
	- 0.07	- 0.35	- 0.42	- 0.41	Difference
Agra Local.....	1.80	1.00	1.06	0.57	Net assimilation in gm »
	1.68	1.07	1.13	0.69	Grain yield in gm »
	+ 0.12	- 0.07	- 0.07	- 0.12	Difference
Punjab 9D	1.73	1.24	1.16	0.78	Net assimilation in gm »
	1.50	1.20	1.17	0.83	Grain yield in gm »
	+ 0.23	+ 0.04	- 0.01	- 0.05	Difference
N.P. 52	2.05	1.17	1.16	0.84	Net assimilation in gm »
	1.74	1.26	1.33	0.92	Grain yield in gm »
	+ 0.31	- 0.09	- 0.17	- 0.08	Difference
CPH 47	1.79	0.87	1.30	0.70	Net assimilation in gm »
	1.71	1.07	1.41	0.89	Grain yield in gm »
	+ 0.08	- 0.20	- 0.11	- 0.19	Difference

C, control; D, defoliation; S, shading; D + S, defoliation + shading.

	For grain yield	For net assimilation
Critical difference at 5 % probability	0.15	0.25
Critical difference at 10 % probability	0.13	0.21

points we may compare, in the first instance, variation in grain yield and net assimilation of the different varieties under the four treatments (c.f. table 3). Although the grains appear to be largely filled by the dry matter assimilated during the post-ear emergence period, as has also been observed by Miller (1939) in the case of winter wheat and by Sayre (1948), in maize, it may be noted that under control the varietal differences in grain yield are not strictly proportional to similar differences in net assimilation.

For instance, the net assimilation in N.P. 52 was the highest in the control and yet its grain yield was not different from that of Agra Local, CPH 47 and N.P. 710. In N.P. 710 the net assimilation was significantly lower than that of N.P. 52, Agra Local and CPH 47 and yet its grain yield was as good as that of these three varieties, obviously due to extraction of dry matter from the reserve material in the shoot. Under all the other three treatments, N.P. 710 showed this high capacity to extract material from the reserves. The grain yield of the other five varieties was substantially lower than the net assimilation under the control. Under the other three treatments, however, reserve material was utilized for grain filling in these five varieties. In Punjab 9D this capacity appeared to be the lowest on the whole.

It will be seen that net assimilation as well as grain yield decreased under the treatments. To see how far these are related, the order of varieties in respect of grain yield and assimilation is indicated in table 4.

Table 4.
Net assimilation

Control	N.P. 52	Agra Local	CPH 47	Punjab 9D	N.P. 165	N.P. 710
Shading	CHP 47	N.P. 165	N.P. 52	Punjab 9D	Agra Local	N.P. 710
Defoliation	Punjab 9D	N.P. 52	N.P. 165	Agra Local	N.P. 710	CPH 47
Shading + Defoliation	N.P. 52	Punjab 9D	CPH 47	N.P. 165	Agra Local	N.P. 710

Grain yield

Control	N.P. 52	CPH 47	N.P. 710	Agra Local	Punjab 9D	N.P. 165
Defoliation	N.P. 710	N.P. 52	Punjab 9D	CPH 47	Agra Local	N.P. 165
Shading	CPH 47	N.P. 52	N.P. 165	Punjab 9D	N.P. 710	Agra Local
Shading + Defoliation	N.P. 52	N.P. 165	CPH 47	Punjab 9D	N.P. 710	Agra Local

There is a rough relationship between the variations in grain yield and net assimilation under the three treatments. To elucidate this relation further the effects of the treatments may be examined on grain number per ear and 1000 grain weight (cf. table 5).

Table 5.

Variety	Grain number per ear				1000 grain weight			
	C	D	S	D + S	C	D	S	D + S
N.P. 165	34.9	30.8	32.6	27.2	43.5	36.2	39.0	30.9
N.9. 710.....	41.0	38.0	36.1	33.7	42.7	35.6	34.4	25.9
Agra Local	37.9	36.4	36.7	34.5	44.4	31.8	32.7	22.2
Punjab 9D	35.7	33.5	31.8	31.7	44.0	37.6	38.8	27.9
N.P. 52	47.1	40.8	40.1	36.7	38.6	32.5	34.6	26.8
CPH 47	45.6	39.1	42.5	38.8	40.3	30.4	36.2	26.0

Critical Difference:

5 % probability	3.2	2.7
10 % >	2.7	2.3

Both grain number and 1000 grain weight are reduced by the treatments. Evidently on the commencement of the treatment competition among grains (which were already set) for carbohydrate set in and some grains increased in size at the expense of others and thus grain number was reduced. It may be supposed that grain number in a variety, the photosynthetic contribution of the ear of which to net assimilation and grains was relatively high, would decrease to a comparatively smaller extent when photosynthesis in the other parts viz., leaf and stem, was restricted or checked. It may also be reasonably assumed that the competition would be less severe in a variety with a smaller grain number. The varietal differences in grain number and grain size may now be considered from this point of view.

Defoliation

Table 6.

Varieties	Punjab 9D	N.P. 52	N.P. 165	Agra Local	N.P. 710	CPH 47
Absolute contribution by ear to net assimilation	0.50	0.45	0.37	0.46	0.55	0.29
Absolute contribution by stem to net assimilation	0.74	0.72	0.65	0.44	0.38	0.58
Grain number per ear	33.5	40.8	30.8	36.4	38.0	39.1
Reduction in grain number	2.2	6.3	4.1	1.5	3.0	6.5
% > > > >	6.0	13.0	12.0	4.0	7.0	14.0
1000 grain weight in gm	37.6	32.5	36.2	31.8	35.6	30.4
Reduction > > >	6.4	6.1	7.3	12.6	7.1	9.9
% > > >	14.0	15.0	17.0	29.0	17.0	25.0

The absolute contribution by the ear and stem to net assimilation was calculated by multiplying the net assimilation (under defoliation) by the respective, calculated ratios of percentage contributions of the ear and stem to total assimilation less the percentage contribution due to leaf. For example, the net assimilation under defoliation in the case of Punjab 9D was 1.24 gm and this multiplied by 29.8/100—26 (vide table 1) gave the absolute contribution by photosynthesis in the ear.

The net assimilation of the varieties Agra Local, CPH 47 Punjab 9D and N.P. 165 was similar under control. Under defoliation, however, the net assimilation of Punjab 9D was higher than that of the other three varieties and so also was the yield. Comparing Punjab 9D and Agra Local the more or less similar percentage reduction in grain number in both may be attributed to similarity in the photosynthetic contribution by the ear and the considerable reduction in grain size in Agra Local to the smaller contribution from the stem.

The greater reduction in grain number in N.P. 165 as compared to Punjab 9D may also be due to the smaller contribution by the ear.

The stem contributions were more or less identical in both these varieties and so also the reductions in 1,000 grain weight.

The greater reduction in grain number and grain size in CPH 47 as compared to Punjab 9D was obviously due to the much smaller contribution by the ear and the stem respectively.

The grain number in N.P. 710 and Punjab 9D was reduced to the same extent due to more or less identical contribution by the ear. The 1,000 grain weight was, however, not reduced in N.P. 710 in spite of much smaller stem contribution obviously due to the fact that N.P. 710 utilized reserves to a considerable extent.

The reduction in grain number in Punjab 9D was less in comparison to N.P. 52 in spite of almost similar contribution by the ear probably because the absolute grain number in the former at the time the treatment commenced was smaller and thus involved less severe competition.

It is not clear why the grain number in CPH 47 suffered to the same extent as in N.P. 52 in spite of the fact that its ear contribution was much smaller. It may be recalled, however, that CPH 47 drew considerably more upon reserves in this case.

The net assimilation (under defoliation) was larger in N.P. 52, although not significantly so, than in Agra Local and N.P. 165 and as the former also drew upon reserves to a slightly greater extent the difference in yield (in favour of N.P. 52) attained a significant level. The smaller reduction in grain number in Agra Local and N.P. 165, (as compared to N.P. 52) in spite of more or less similar contribution by the ear was perhaps due to the

smaller number of grains involving less severe competition. The contribution by the stem was, however, smaller in Agra Local as compared to N.P. 52 and N.P. 165 and thus its grain size suffered more.

N.P. 710 also assimilated less than N.P. 52 under defoliation but its grain yield was equal to that of the latter due to much greater extraction of dry matter from the reserves.

To sum up: Varieties the net assimilation of which suffered comparatively less under defoliation, yielded on the whole better. Varieties, like N.P. 710, may occur whose grain yield may not suffer in proportion to reduction in net assimilation on account of their ability to extract more of the reserve material. The proportions of contributions by the ear and the stem appear to determine to some extent the number of mature grains and their size.

Shading

CPH 47 outyielded N.P. 165 and Punjab 9D because its net assimilation was higher, although not significantly so, and it also utilized more of reserve material in grain filling. N.P. 52 outyielded Punjab 9D because the latter hardly drew anything from the reserves. CPH 47 was superior to Agra Local and N.P. 710 in respect of both net assimilation and grain yield. N.P. 52 outyielded Agra Local because the latter drew much less from the reserves and N.P. 710 because of very low net assimilation in the latter variety. N.P. 710, in spite of its lowest net assimilation under this treatment, yielded as well as N.P. 165, Punjab 9D and Agra Local because of its comparatively high capacity to draw upon the reserves.

To sum up: Yielding capacity under this treatment appeared to be determined by both the net assimilation of the shoot and the ability to utilize reserves. On shading the ear, grain number was reduced very likely due to competition setting in between grains for dry matter and the extent of this reduction would seem to depend upon the rate of transport to the ear from the shoot. Under 'defoliation' it was possible to account for the percentage reduction in grain number on the basis of difference in the photosynthetic contributions by the ear and the stem respectively. Data at hand do not permit of an estimate of the rate of transport from the shoot to the ear, in the different varieties, under 'shading' and it is, therefore, not possible to account for the differences in grain number and size between the varieties.

Shading plus Defoliation

It will be seen that the grain yields are fairly correlated with net assimilation. Again N.P. 710 yielded as well as the others, in spite of the lowest net

assimilation, because it extracted considerably more from the reserves. The differences in grain number and grain size between the varieties cannot be accounted for, for the reason stated above.

Discussion

Under control grain yield appears to be, on the whole, less than the dry matter assimilated during the post-ear emergence period (exception, N.P. 710). Apparently grain size and, therefore, factors concerned with grain development (and presumably of non-carbohydrate nature) govern the utilization of the assimilate under such conditions. Gustafson and Stoldt (1936) and Bald (1946) also arrived at a similar conclusion. Gustafson and Stoldt (1936) observed that on increasing food producing area fruit size in tomato can be increased considerably, although not proportionately and they ascribed this lack of proportionality to factors other than nutrition at the time of enlargement influencing ultimate fruit size. According to Bald (1946), the basic efficiency of metabolic centres is similar for different varieties of domestic potato and that under similar environmental conditions division of metabolites in various proportions between organs induces in different varieties and strains differences in growth, maturity and yield. This partition of metabolites according to Bald, is governed by hormones in leaves or growing tips. The results of the observations under conditions that retard photosynthesis and reported in this paper, do not, however, warrant an unqualified acceptance of Bald's generalisation. This lack of agreement does not, however, imply any criticism of Bald's view because it refers to the potato plant only. It should also be noted that tillering as a factor in yield has not been taken into consideration in this preliminary analysis of the problem.

In the event of restricted photosynthesis in the shoot (after ear emergence), N.P. 710, among the six varieties, would appear to suffer the least in yield because reserves in the stem and the ear by its own photosynthetic activity, contribute a substantial proportion to the dry matter of the grain. In Punjab 9D the reserves are utilized to a very small extent and the assimilate from the stem contributes much more to the dry matter of the grain than that derived from the leaf. It may, therefore, be expected to yield less than N.P. 710 under this condition. N.P. 52 is more or less of the same type as Punjab 9D but its absolute assimilation is the highest and it also utilizes reserves to a greater extent than Punjab 9D. One may, therefore, expect it to yield better than Punjab 9D and perhaps as well as N.P. 710. CPH 47 and N.P. 165 differ from N.P. 52 and Punjab 9D in that both leaf and stem contribute

equally to the dry matter of the grain; reserves are, however, utilized to the same extent as in N.P. 52 but in view of the comparatively smaller contribution by the ear to the dry matter of the grain their yields may be expected to be lower than those of N.P. 52 and Punjab 9D. In Agra Local the contribution by the leaf to the dry matter of the grain is more or less similar to that of N.P. 52 but the contribution by the stem is much smaller; as compared to Punjab 9D its grain is filled relatively more by the leaf assimilate than by the stem assimilate. It may, therefore, be expected to yield as well as Punjab 9D but perhaps less than N.P. 52 because the assimilation capacity of the latter is very high. Under such conditions the varieties may, therefore, be expected to yield in the following order: -

<u>N.P. 710</u>	<u>N.P. 52</u>	<u>Punjab 9D</u>	<u>Agra Local</u>	<u>N.P. 165</u>	<u>CPH 47</u>
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We may now compare the order of grain yield actually obtained in an experiment undertaken during the same season and where the transport of the assimilate from the shoot to the ear was deliberately checked. Five varieties viz., N.P. 710, N.P. 52, N.P. 165, Punjab 9D and Agra Local were subjected to treatments: control, defoliation, puncturing the peduncle (12 punctures with a needle and plugged with paraffin) and combination of defoliation and puncturing. Only mother shoots were used for the experiment and the treatments were commenced 5 days after anthesis. All the four treatments were included in one pot and there were ten such pots for each variety. Only grain yields were recorded and these are given in table 7.

The order of varieties as regards yield, under control, in this experiment does not exactly tally with the same in the first experiment. While no explanation can be given for this it may be noted that these plants were grown in pots of smaller size and that the tillers were not removed. The order of varieties as regards yield, when the peduncle was punctured, appears

Table 7. Grain yield in gm per shoot.

Control	N.P. 52	Punjab 9D	N.P. 165	N.P. 710	Agra Local
	1.72	1.69	1.64	1.57	1.49
Puncturing	N.P. 52	N.P. 710	Punjab 9D	Agra Local	N.P. 165
	1.25	1.24	1.03	1.01	0.80
Defoliation	N.P. 52	Punjab 9D	N.P. 710	Agra Local	N.P. 165
	1.55	1.36	1.26	1.26	1.13
Puncturing + Defoliation	N.P. 52	Punjab 9D	N.P. 710	Agra Local	N.P. 165
	1.19	1.12	1.02	0.92	0.66

Critical difference at 5 % probability, 0.16.

Critical difference 10 % probability, 0.13.

to be according to expectation and the influence of defoliation is also more or less similar to that obtained in the first experiment. The rank of Punjab 9D under the combined defoliation and puncturing treatment is anomalous — all the other varieties gave lower yields under this treatment as compared to 'puncturing', and obviously the higher yield in Punjab 9D is due to sampling error.

It is tempting to speculate upon the bearing of these observations on the resistance of varieties to rust. If the incidence of leaf or stem rust, after ear emergence, mainly involves interference with photosynthesis in the leaf and stem and transport of the assimilate to the ear, then the comparative resistance (as judged by yield) of some varieties may perhaps find an explanation along the lines discussed above. The criterion of rust resistance usually, and quite rightly, adopted is the extent of the spread of the symptoms under an artificial epidemic. It sometimes happens that new races of rust arise by mutation and the varieties immune to the older races no longer prove resistant to these. While the usefulness of varietal tests by means of artificial inoculation is accepted, it is tentatively suggested that attempts to combine high capacity to utilize reserves, high total assimilation rate and high assimilation rate of the ear, might prove useful for inducing rust resistance.

It is interesting to note in this connection that the yield of variety N.P. 710 was found to be very promising as a result of varietal trials carried out at several centres in the country during the 1946–47 season when a serious rust epidemic occurred and N.P. 710 was also infected. We are grateful to Dr. B. P. Pal for this information.

Whether these characters can be genetically combined and whether, in fact, they are genetically determined are questions that might repay investigation. On a priori grounds it appears that presence of these characters, or of at least one of them, would give a variety a definite advantage in yielding capacity in case of epidemics of such intensity as reduce yield but do not entirely destroy the crop. The isolation of suitable indices of these characters, as an aid to the breeder, also demands attention. What is the varietal difference in the assimilation rate of the shoot and the ear due to?

Summary

As a preliminary step in the physiological analysis of yield in wheat, varietal differences in the contributions from photosynthesis in the different organs (during the post-ear emergence period) and from reserve material in the stem to the dry matter of the grain have been investigated.

Six varieties of vulgare wheats viz., N.P. 165, N.P. 710, N.P. 52, Punjab 9D, Agra Local and CPH 47 were included in this study.

The experimental treatments which commenced 5 days after anthesis consisted of control, defoliation, shading of ear and a combination of shading of ear and defoliation. Only mother shoots (20 per variety) were subjected to these treatments and the tillers were removed. Both initial and final (harvest) plant samples were collected and the changes in the dry matter of the leaf, stem with sheaths, ear and chaff were estimated. From the mean effects of defoliation and shading of ear on the changes in the dry matter an estimate of the contribution by photosynthesis in the leaf and ear respectively to the dry matter of the grain was worked out. The effect of combined treatments gave an estimate of the contribution by photosynthesis in the stem to the dry matter of the grain.

Grain number as well as grain size was reduced, in comparison with the control, under all the other three treatments. There was some indication that under defoliation the reduction in grain number was comparatively less in the varieties in which the photosynthetic contribution by the ear was high.

Under control, grains were filled very largely by the dry matter derived from assimilation during the experimental period (exception N.P. 710). In N.P. 52 the net assimilation of dry matter was the highest during the experimental period and the lowest, on the whole, in N.P. 710. The varietal differences in grain yield were not, however, proportional to the same in net assimilation. Apparently factors of non-carbohydrate nature are concerned with differences in grain yield.

Under treatments that checked the photosynthetic activity of the different organs the relation between grain yield and net assimilation appeared, however, more clear.

In N.P. 710 reserves in the stem and photosynthesis in the ear contributed substantially to grains; in N.P. 52 the net assimilation was the highest and reserves in the stem were utilized to a greater extent as compared to Punjab 9D but to a smaller extent as compared to N.P. 710; in Agra Local reserves from the stem were utilized to a smaller extent than in N.P. 710 and the net assimilation was lower as compared to that of N.P. 52; in N.P. 165 and CPH 47 the photosynthetic contribution by the ear was, on the whole, lower. It is, therefore, concluded that when photosynthesis in the shoot is checked the varieties would yield in the order:

$$\text{N.P. 710} = \text{N.P. 52} > \text{Punjab 9D} = \text{Agra Local} > \text{N.P. 165} = \text{CPH 47}$$

The above conclusion was checked in a slightly different way by another experiment which included as treatments: control, puncturing the peduncle,

defoliation and a combination of the last two. The object of puncturing the peduncle was to restrict the supply of assimilate from the leaf and the stem to the ear. The varieties yielded in the same expected order when the peduncle was punctured.

The bearing of these observations on the problem of rust resistance is briefly indicated and it is tentatively suggested that under a not very severe rust epidemic varieties with a high capacity to draw upon the reserve material in the stem, and high total assimilation capacity or high assimilation capacity of the ear might be expected to yield relatively better.

In conclusion the authors wish to record their thanks to Dr. B. P. Pal, Head of the Division of Botany, for suggesting the choice of varieties and for his inspiration and continual interest in the progress of the work. Our thanks are also due to Mr. P. N. Saxena, Assistant Statistician, for guidance in the statistical analysis of the data.

References

- Archbold, H. K.: Physiological studies in plant nutrition XIII. Experiments with barley on defoliation and shading of the ear in relation to sugar metabolism. — *Ann. Bot.*, N.S. 6: 487. 1942.
- Asana, R. D. & Mani, V. S.: Photosynthesis in the ears of five varieties of wheat. — *Nature* 163: 451. 1949.
- Bald, J. G.: A plan of growth, maturity and yield of the potato plant. — *Emp. J. Exp. Agr.* 14: 43—48. 1946.
- Balls, W. L. et al: Analysis of agricultural yield. — *Phil. Trans.* 206 B.: 103, 403. 1915. 208 B: 157. 1918.
- Boonstra, A. E. H. R.: Difference in vitality of the leaves of four varieties of oats as connected with the yield. — *Meded. Landb. Hooges. Wageningen.* 33: 1. 1929.
- Physiological research in the service of plant breeding. — *Meded. Landb. Hooges. Wageningen.* 38: 1. 1934. (Plant Breed. Abst. 5, item 612, 1934—35.)
- Plant Breeding and Plant Physiology. — *Der Züchter*, 3: 345. 1931. (Plant Breed. Abst. 2, item 207, 1931—32.)
- Crowther, F.: Studies in growth analysis of the cotton plant under irrigation in the Sudan. I. The effect of different combinations of nitrogen applications and water supply. — *Ann. Bot.* 48: 877. 1934.
- Third conference on cotton growing problems. Report and summary of proceedings. P. 61. 1938.
- Gregory, F. G.: Conference of Empire Meteorologists, Agric. section II. Papers and Discussions. P. 29. 1929.
- Gustafson, F. G. & Stoldt, E.: Some relations between leaf area and fruit size in tomatoes. — *Plant Physiol.* 11: 445. 1936.
- Knowles, F. & Watkin, J. E.: The assimilation and translocation of plant nutrients in wheat during growth. — *J. Agr. Sci.* 21: 612. 1931.
- Miller, E. C.: A physiological study of the winter wheat plant at different stages of its development. — *Tech. Bull.* 47, Agric. Expt. Sta. Kansas. 1939.
- , Gauch, H. G., & Gries, G. A.: A study of the morphological nature and physio-

- logical functions of the awns of winter wheats. — Kansas Agric. Exp. Station Tech. Bull. 57: 1. 1944.
- Sayre, J. D.: Mineral accumulation in corn. — Plant Physiol. 23: 267. 1948.
- Smith, H. F.: The physiological relations between tillers of a wheat plant. — J. Counc. Sci. Indust. Res. Aust. 6: 32. 1933.
- Vervelde, G. H.: On the role of awns in cereals. — Meded. Landb. Hoogeschool Wageningen. 48: 35. 1946. (Field crop Abst., 2, item 202, 1949.)
- Watson, D. J. & Norman, A. G.: Photosynthesis in the ear of barley and the movement of nitrogen into the ear. — J. Agric. Sci. 29: 321. 1939.
- Went, F. W.: Plant growth under controlled conditions. III. Correlation between various physiological processes and growth in the tomato plant. — Am. J. Bot. 31: 597. 1944.
- Worzella, W. W.: Some objects in breeding for yield and other agronomic characters in wheat. — J. Am. Soc. Agr. 33: 174. 1941.

Effect of α -Naphthaleneacetic Acid on Dichotomous Branching of Isolated Roots of *Pinus silvestris* (A Preliminary Report)

By

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As has been shown in an earlier paper (Slankis 1948), dichotomous branching may be produced in isolated pine roots by addition of exudates of mycorrhizal fungi (e.g. species of *Boletus*) to the nutritive solution. Similar branching of pine roots occurs in nature, according to the investigations of Melin (1923) and others, in connection with the formation of mycorrhiza. Further investigations (Slankis 1949) indicated that β -indoleacetic acid might be one of the components of the fungous exudates which cause this branching. β -indoleacetic acid which is known to have a marked effect on root formation (Thimann 1948), produced in isolated pine roots not only a rich development of short roots but also dichotomous branching of the last roots formed. The question now arises: Do other substances of the so-called »auxins» which have a strong influence on root-formation (Thimann l.c.) produce a similar branching? Up till now experiments have been made only with α -naphthaleneacetic acid which, as is generally known (Zimmerman and Wilcoxon 1935, Zimmerman and Hitchcock 1935 and others), can produce a rich formation of adventitious roots, as well as enhance the branching of the root system.

Five-months old pine roots were used in the experiments. The seeds had been collected from Uddheden (Värmland, Sweden), about 110 m altitude. The method of preparing the root cultures has previously been described (Slankis 1948 a, p. 278). The nutrient solution contained the following organic and inorganic substances per litre redistilled water: 71,95 mg $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 20,47 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg KH_2PO_4 , 1,66 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (according



Fig. 1. Isolated pine roots, 6 $\frac{1}{2}$ months old, cultivated in nutrient solution without α -naphthaleneacetic acid. $\times 1.6$.

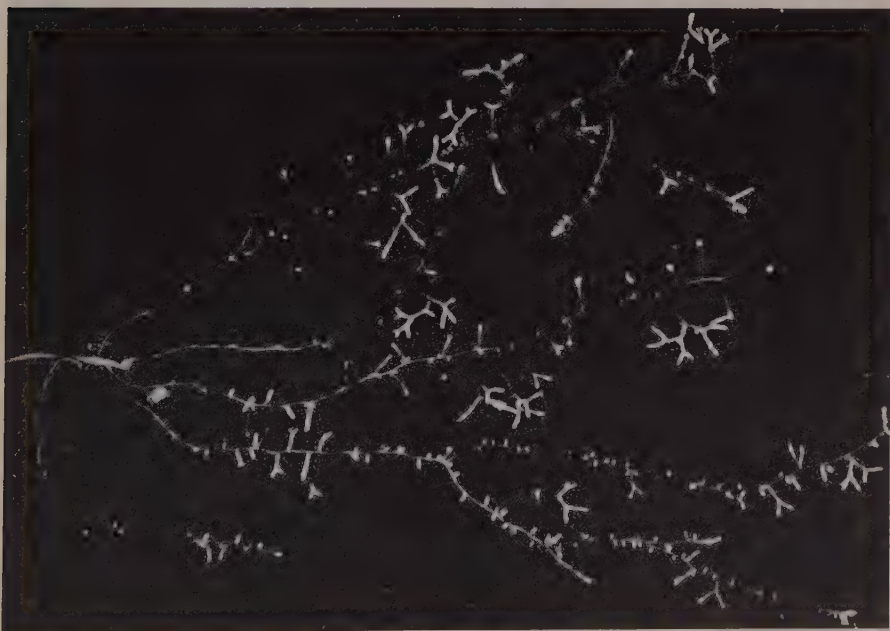


Fig. 2. Isolated pine roots, 6 $\frac{1}{2}$ months old, maintained during the last 45 days in nutrient solution containing 30 γ / 20 ml α -naphthaleneacetic acid. $\times 1.6$.

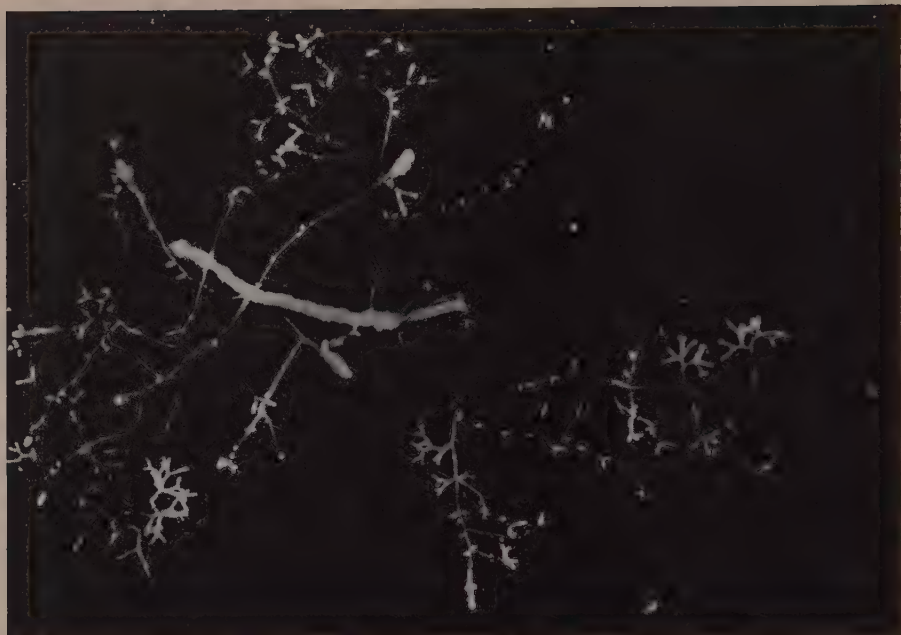


Fig. 3. *Isolated pine roots, 6 1/2 months old, maintained during the last 45 days in nutrient solution containing 50 γ / 20 ml α-naphthaleneacetic acid. × 1.6.*

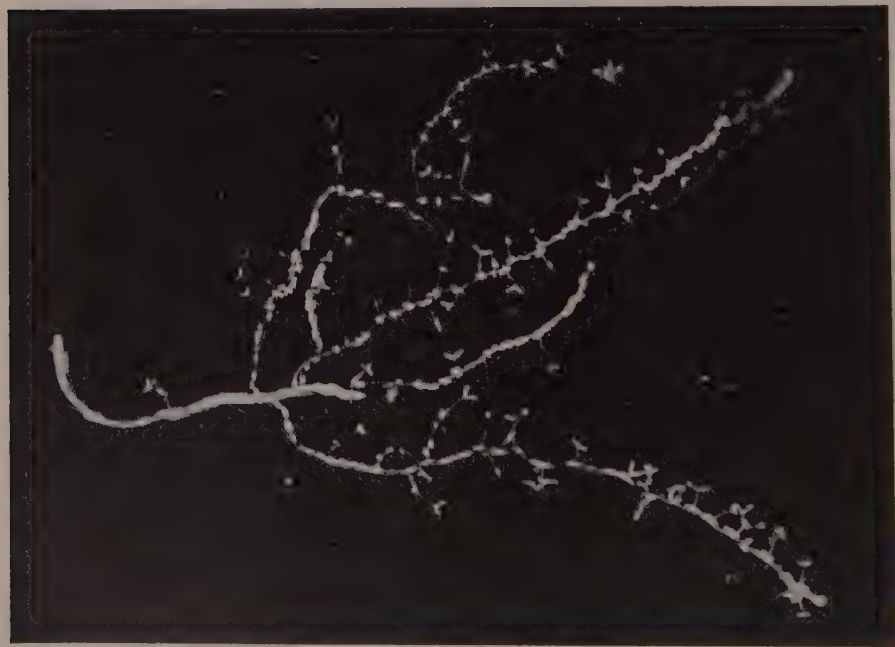


Fig. 4. *Isolated pine roots, 6 1/2 months old, maintained during the last 45 days in nutrient solution containing 70 γ / 20 ml α-naphthaleneacetic acid. × 1.6.*

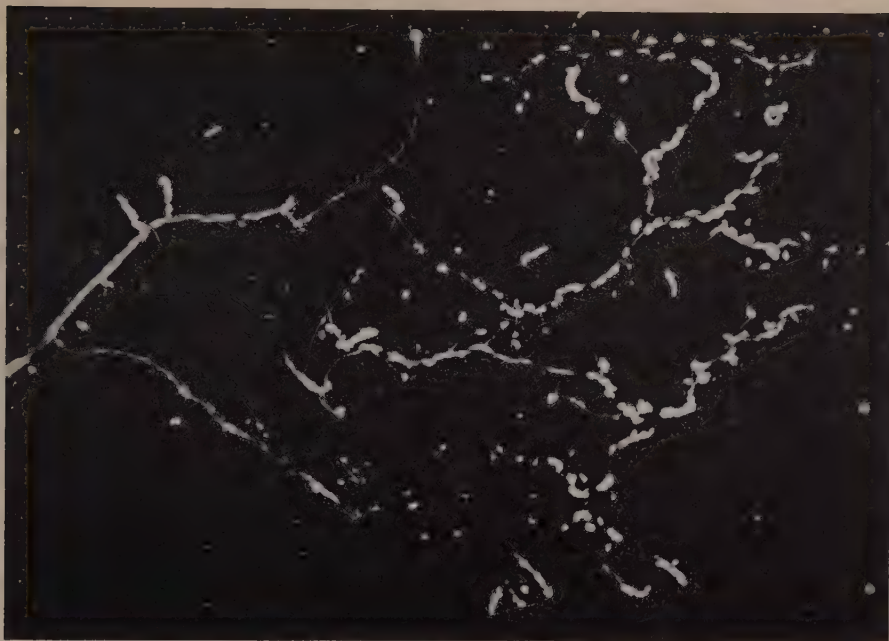


Fig. 5. Isolated pine roots, 6½ months old, maintained during the last 45 days in nutrient solution containing 200 γ / 20 ml α -naphthaleneacetic acid. $\times 1.6$.

to Robbins and White 1936) and 0,5 ml »A—Z» solution according to Hoagland (Schropp and Scharer 1933, p. 544); 7 % sucrose (Merck-Rahway, N. J.) as a carbon source (cf. Slankis 1948 a), as well as 50 γ thiamin-HCl and 500 γ cholinechloride (both from Hoffmann-La Roche). Each 100 ml Erlenmeyer flask contained 20 ml of nutrient solution, autoclaved for 15 minutes at a pressure of 1,2 atm. α -naphthaleneacetic acid was added to the flasks in one of following quantities: 0,1 γ , 1 γ , 10 γ , 20 γ , 30 γ , 50 γ , 70 γ , 100 γ and 200 γ . The flasks containing the roots were incubated at a temperature of 19° C (cf. Slankis 1949 a). The nutrient solution with the corresponding quantity of α -naphthaleneacetic acid was renewed every 15 days.

In the course of 45 days, the following changes in the roots were observed:

1) As in the case of β -indoleacetic acid, α -naphthaleneacetic acid produced, at certain concentrations, a dichotomous branching of the short roots (Figs. 1—4). Dichotomy of the roots occurred in flasks containing α -naphthaleneacetic acid in quantities of 1 γ to 70 γ . At amounts of 1 γ to 50 γ , forking began after 7 to 8 days, the roots branching repeatedly in many cases. At 70 γ , repeated dichotomous branching was no longer observed (Figs. 2—4). It appears that α -naphthaleneacetic acid has the greatest influence at quan-

tities ranging from 10 γ to 50 γ . No dichotomous branching was observed at 100 γ or 200 γ of α -naphthaleneacetic acid. On the other hand, in these cases the tips of the short roots showed various rounded swellings (Fig. 5). Similar formations were also noticed in the short roots at lower quantities, but these were not numerous (Figs. 2—4).

2) It has been shown previously that the appearance of the forks produced by β -indoleacetic acid, depends upon the concentration of this compound. A similar effect was obtained by α -naphthaleneacetic acid. Thus, as the concentration of α -naphthaleneacetic acid increased, the branches of the forks became shorter (Figs. 2—4).

3) As with β -indoleacetic acid, so the addition of α -naphthaleneacetic acid caused a rich formation of short roots. At 1 γ to 30 γ α -naphthaleneacetic acid, many short, solitary roots developed. At 50 γ these roots formed together in rows so closely as to produce a 'comb-like' effect. At 70 γ the number of solitary and 'comb' roots decreased and in their place rows of swellings developed. Each swelling probably consists of a number of short roots, as indicated by the wavy outline of the surface. Generally at 100 γ and 200 γ , similar swellings also appeared. These often differed from the former, in being larger and in lacking the wavy surface (Figs. 4—5).

References

1. Melin, E.: Experimentelle Untersuchungen über die Konstitution und Ökologie der Mykorrhizen von *Pinus silvestris* L. und *Picea Abies* (L.) Karst. — *Mykolog. Untersuch. u. Ber. von R. Falk* 2: 73. 1923.
2. Schropp, W. & Scharrer, K.: Wasserkulturversuche mit der 'A—Z-Lösung' nach Hoagland. — *Jahrb. wiss. Bot.* 78: 544. 1933.
3. Slankis, V.: Einfluss von Exudaten von *Boletus variegatus* auf die dichotomische Verzweigung isolierter Kiefernwurzeln. — *Physiol. Plant.* 1: 390. 1948.
4. — Verschiedene Zuckerarten als Kohlehydratquelle für isolierte Wurzeln von *Pinus silvestris*. — *Physiol. Plant.* 1: 278. 1948 (a).
5. — Wirkung von β -Indolylessigsäure auf die dichotomische Verzweigung isolierter Wurzeln von *Pinus silvestris*. — *Svensk Bot. Tidskr.* 43: 603. 1949. (Preliminary report.)
6. — Einfluss der Temperatur auf das Wachstum der isolierten Wurzeln von *Pinus silvestris*. — *Physiol. Plant.* 2: 131. 1949 (a).
7. Robbins, W. J. & White, V. B.: Limited growth and abnormalities in excised corn root tips. — *Bot. Gaz.* 98: 209. 1936.
8. Thimann, K. V.: Plant growth hormones. — G. Pincus & K. V. Thimann: The hormones 1: 5. Academic Press Inc. Publishers, New York. 1948.
9. Zimmerman, P. W. & Wilcoxon, Fr.: Several chemical growth substances which cause initiation of roots and other responses in plants. — *Contrib. Boyce Thompson Inst.* 7: 209. 1935.
10. Zimmerman, P. W. & Hitchcock, A. E.: The response of roots to 'rootforming substances'. — *Contrib. Boyce Thompson Inst.* 7: 439. 1935.

The Permeability of Nitella Cells to Rapidly Penetrating Non-Electrolytes

By

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Introduction

As early as 1899, Overton, when classifying the different organic compounds according to their ability to penetrate living protoplasts, stated that the most rapidly penetrating compounds include all the monohydric alcohols, the corresponding aldehydes and ketones, the nitroalkyles, alkylcyanides, ethers, and the esters of inorganic and organic acids (insofar as the last-named do not contain more than one hydroxyl group). All these compounds were found by him to permeate about as rapidly as water. A further differentiation of these substances as regards their permeation power was not given.

During the fifty years that have passed since the fundamental work of Overton, the permeability of living cells to more slowly penetrating non-electrolytes has been thoroughly studied by numerous investigators. The above-mentioned group of the most rapidly penetrating substances has, on the other hand, owing to technical difficulties, been rather neglected.

Of the few investigations pertaining to this group of substances those of Holdheide (1930, 1931), Zehetner (1934), and Wartiovaara (1944, 1949) are the most important.

Holdheide found that plant cells often burst, owing to a sudden increase of their internal pressure, when put into excessively high concentrations (about 20—100 %) of methanol, ethanol, or some other substances which are highly soluble both in water and in lipoids.

Zehetner corroborated these observations and found, by measuring the volume changes of plasmolyzed protoplasts which occur when the cells are transferred

from a solution of, say, sugar to an equimolar sugar solution also containing some alcohol, that the alcohol causes some kinds of protoplasts to contract and others to expand. It thus seems fairly obvious that some plant protoplasts are more permeable to water than to alcohol while others are, on the contrary, more permeable to alcohol than to water. A sudden change from the former type to the latter was also observed.

The first truly quantitative determinations of the permeation power of substances belonging to this group were performed by Wartiovaara, who measured experimentally the permeability of *Tolypellopsis* and *Nitella* cells to heavy water and to four monohydric alcohols. From his experiments he also calculated the permeability of the plasma membrane alone to these substances.

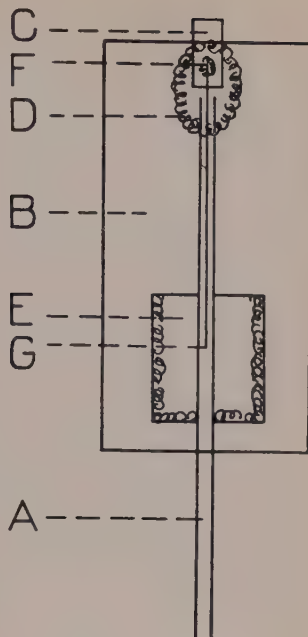
In view of how little is known, so far, about the permeation of the most rapidly penetrating non-electrolytes it is perhaps justifiable to publish the following observations in spite of their only semi-quantitative character.

Methods

If a cell which is saturated with water is placed in a solution of a substance which does not penetrate the surface layers of the cell as easily as water, the cell will contract owing to the osmotic withdrawal of water from it. If the solute then gradually enters the cell by diffusion, this causes the cell to expand again until it eventually reaches its original volume. If the solution surrounding the cell is now replaced by water, the cell will first swell owing to the uptake of water, and then gradually contract to its original volume owing to the diffusing out of the solute previously taken up. By watching these volume changes it should thus be possible to ascertain how rapidly the solute in question enters and leaves the cell and also how permeable to water the cell is. As compared with the plasmolytic method of permeability determination, this so-called turgor tension method has the advantage that no hypertonic solutions need be used, the danger of abnormal permeability changes being thus greatly reduced. Another advantage, connected with the first-named one, is that the same cell can be used for numerous successive permeability determinations.

Perhaps the most promising material for experiments of this kind are the giant internodal cells of Characean plants. Until now they seem to have been used in this way only by Collander and Bärlund (1933, pp. 63—71) and by Saubert (1937), who both worked with »leaf» cells of *Chara ceratophylla*. Owing to their excessive thickness (about 1.2—1.3 mm.) these cells are not, however, ideal for the study of very rapidly permeating substances, because the diffusion resistance in the sap of such cells might well be a factor limiting the rapidity of the permeation process.

Fig. 1. Schematic sketch of the experimental device.
 A glass capillary, B glass slide, C piece of a cover-slip,
 D ring of wax+cocoa butter, E cover-slip, F fixed end
 of the cell, G free end of the cell.



The experiments to be described here were therefore carried out with cells of *Nitella mucronata* only 0.35—0.50 mm. thick and 20—70 mm. long. The osmotic concentration of the cell sap corresponded to that of a 0.25—0.30 molar solution of a non-electrolyte.

In order to be able to watch continually the contraction and elongation processes of the cells the following procedure was used. (Cf. Fig. 1.)

A glass capillary (A), about 2.3 mm. thick and 100 mm. long, is fixed with bees-wax onto a glass slide (B) and filled with water. The cell to be studied is then fixed by one end onto a rectangular piece (C) of cover-slip, using a mixture of 1 part of bees-wax and 4 parts of cocoa butter. The cell is then pushed into the capillary and the adhering cover-slip fixed onto the glass slide. Finally the protruding end of the cell is encircled by a ring (D) of the same mixture, and a cover-slip (E) fixed with bees-wax and vaseline on the glass slide so as to cover that part of the capillary in which the free end of the cell is located. The space between the glass slide and the cover-slip is filled with water.

The slide is now put onto the microscope-stage so that the free end of the cell is visible in the microscope. The microscope having been brought into a suitably inclined position, a test-tube with its end drawn out to a capillary is fixed by a stand so that its capillary ends about 1 mm. above the encircled area of the glass slide. If water or an aqueous solution is then poured into the test-tube, it flows at a fairly constant speed through both capillaries so that the cell is bathed along its whole length by the fluid. At the same time the changes in length of the cell are observed by watching the movements of an easily discernible point

(e.g., a little epiphyte) near its free end along the micrometer scale of the microscope. Generally a Leitz objective No. 4 (sometimes No. 3) and a Leitz micrometer eye-piece No. 2 were used.

If very rapid changes of length had to be recorded, it was found necessary for the observer to dictate the readings to a second person who noted them down. Using a metronome it was in this way possible to make the observations every two seconds. Mostly, however, the observations were made at longer intervals.

The experiments were carried out at temperatures between 18° and 21° C. Tap-water from which the excess of air had been removed by heating or by evacuation was always used as solvent in preparing the solutions to be used.

A closer study of the *Nitella* cells showed that they possess three properties which render them less suited for experiments of this kind than was originally anticipated.

(1) The contractions of a given cell in different solutions are not directly proportional to the osmotic pressures of the solutions. Thus, if the contraction caused by a 0.1 molar urea solution is taken as 1, the contractions caused by a 0.2 molar solution of the same substance were found, in 17 cells, to vary between 2.6 and 3.3 being on an average 2.9.

(2) It cannot be taken for granted that the volume changes of the cells are exactly proportional to their changes of length.

(3) The length of a given cell does not depend only upon the actual composition of the surrounding solution, but also — at least in many cases — upon whether the cell has previously been made to contract or to expand. If, for example, a cell is first measured in water and is then caused to contract by treatment with a solution of a non-penetrating substance after which it is again treated with water, it often happens that it does not quite reach its original length but remains a little shorter. Correspondingly a cell which has been caused to elongate often remains somewhat longer when brought back into the original medium. This phenomenon, which largely vitiates the exactitude of the permeability measurements, seems to be due to a certain plasticity of the cell wall, sometimes perhaps also to the occurrence of some friction between the cell and the capillary wall.

Results

Permeability to Water. In Fig. 2 the first part of curve A shows the contraction of a *Nitella* cell under the influence of a 0.2 molar urea solution while the second part shows the elongation of the same cell after the urea solution has been replaced by water. Curve B shows the reaction of the same cell towards a 0.2 molar sucrose solution followed by water. It is seen that both the contraction and the elongation processes have a roughly

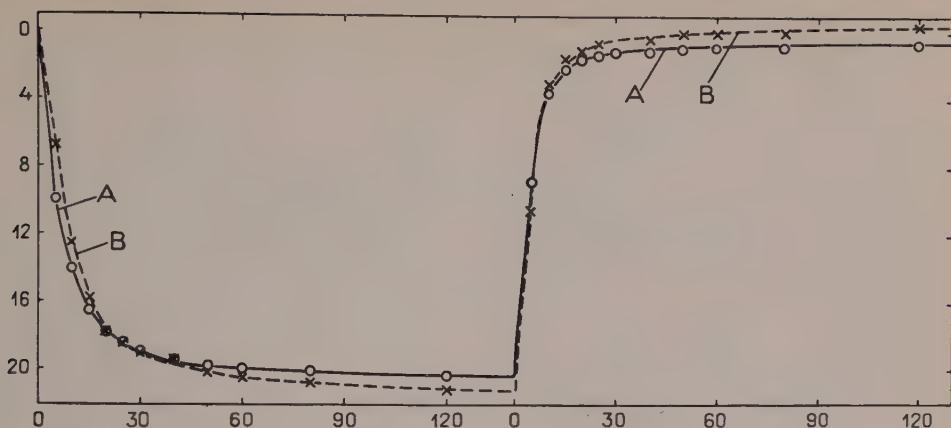


Fig. 2. Contractions of a *Nitella* cell (0.41 mm. thick) caused by 0.2 molar solutions of urea (curve A), viz., of sucrose (curve B) and elongations of the same cells after the solutions have been replaced by tap water. Abscissa: time in seconds. Ordinate: position of the end of the cell on the micrometer scale. Curve A is the mean of 4 experiments, curve B of 2 experiments.

exponential course. The first half of each change of length is accomplished in about 5—8 seconds. On the basis of these data one may try to calculate approximately the permeability of the cell to water using to this end, for instance, the equation (1) of Wartiovaara (1949, p. 187) in a slightly modified form, viz.,

$$P = 62 \frac{d}{t}$$

where P denotes the permeability constant in cm/hour, d the diameter of the cylindrical cell (in mm.), and t the time (in seconds) in which the half quantity of substance has been taken up or given out. In this way we get $P_{\text{water}} \sim 3\text{--}5$ cm/hour. Also in several other experiments of this kind half contraction times, viz., half elongation times, of about 5—10 seconds were obtained corresponding to P_{water} values of about 3—5 cm/hour. Because of the great rapidity of these changes of length which makes an exact measurement difficult and also on account of the sources of error already mentioned, no great stress is laid on these results. They only confirm, in a general way, the findings of Wartiovaara (1944, 1949), according to which the permeability of Characean cells (*Tolypellopsis*, *Nitella mucronata*) to water is very great as compared with the statements in the earlier literature about the permeability of other plant cells to water (cf. Bochsler 1948).

Permeability to Solutes. In order to determine the permeability of the *Nitella* cells to very rapidly penetrating solutes the contractions caused by

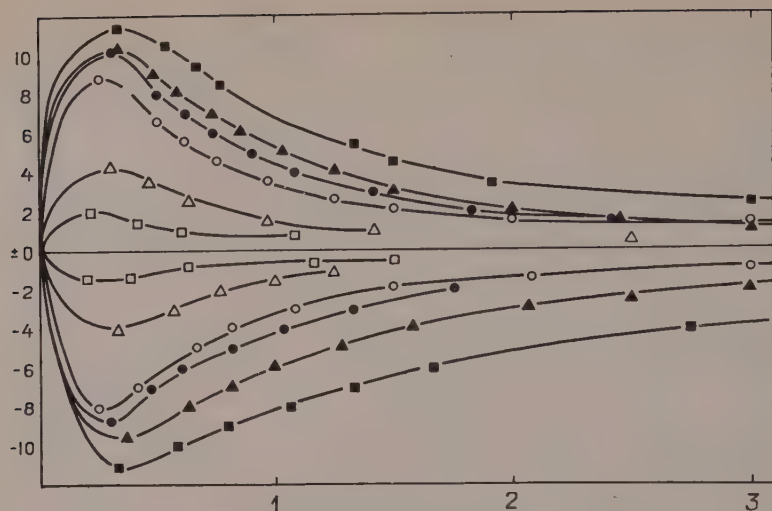


Fig. 3. Contractions of a *Nitella* cell (0.45 mm. thick) caused by 0.3 molar solutions of *n*-butanol (\square), *n*-propanol (\triangle), methanol (\circ), ethanol (\bullet), *iso*-propanol (\blacktriangle), and *tert*-butanol (\blacksquare) and the elongations of the same cell after the solutions have been replaced by water. Abscissa: time in minutes. Ordinate: position of the end of the cell on the micrometer scale.

solutions of different substances were compared with those caused by an equimolar solution of methanol, which was thus used as a kind of standard substance. Fig. 3 gives an example of such an experiment. The curves below the 0 line represent contractions caused by the solutions tested while the curves above that line show the elongations brought about by replacing the solutions by water. It is seen that both the contractions and the subsequent elongations in water increase in the order: *n*-butanol < *n*-propanol < methanol < ethanol < *iso*-propanol < *tert*-butanol. It seems, therefore, highly probable that this is also the order of decreasing permeation power.

Table 1 contains data on the contractions caused by solutions of 28 very rapidly permeating non-electrolytes as compared with those caused by equimolar methanol solutions. Only the heavy water forms an exception here, for, owing to its extremely rapid penetration, it was found necessary to use it as a 10 molar solution while the cells, of course, do not tolerate methanol concentrations nearly so high. The 10 molar solution of deuterium oxide (or, somewhat more properly speaking, a 20 molar solution of HDO) was found to cause contractions which were with respect to those caused by a 0.1 molar methanol solution as about 0.7 to 1.0.

Although the experiments on which Table 1 is based were carried out at different times (partly in April—June 1947 and partly in September 1949—

Table 1. Contractions caused by solutions of rapidly penetrating substances (=contraction I) as compared with the contractions of the same cell caused by equimolar methanol solutions (=contraction II). RC = relative contraction = contraction I / contraction II, MRC = mean relative contraction, M molecular weight, k = partition coefficient ether/water (mostly after Collander 1949). The figures given in parentheses are only estimated. All contractions expressed in micrometer scale units.

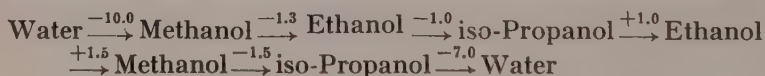
Solute	Molarity	Contraction		RC	MRC	M	k
		I	II				
(1) Deuterium oxide	10	1.8	—	0.007	0.007	20	0.0009
»	»	1.1	—	0.006			
(2) Nitromethane	0.5	0.52	8.0	0.07	0.06	61	> 1
»	0.4	0.88	17.0	0.05			
(3) Acetonitrile	»	0.82	4.0	0.21	0.16	41	0.60
»	»	1.63	17.0	0.10			
(4) Propionitrile	»	0.85	4.0	0.21	0.16	55	(2.4)
»	»	1.65	17.0	0.10			
(5) n-Butanol	0.3	1.85	7.4	0.25	0.25	74	7.7
»	»	1.1	4.6	0.24			
(6) Ethyl Acetate	0.4	1.0	4.0	0.25	0.25	88	8.5
»	0.3	1.15	4.6	0.25			
(7) Ethyl Ether	»	1.2	4.6	0.26	0.26	74	10
»	»	1.2	4.6	0.26			
(8) Methylal	0.5	2.2	8.0	0.28	0.29	76	?
»	0.4	4.4	15.4	0.29			
(9) iso-Butanol	0.3	1.7	4.6	0.37	0.32	74	6.9
»	0.4	4.1	15.4	0.27			
(10) Acetone	0.3	2.1	4.45	0.47	0.35	58	0.62
»	»	1.2	3.8	0.32			
»	0.5	2.2	8.0	0.28	0.35	58	0.62
»	0.3	1.5	4.6	0.33			
»	0.4	1.4	4.0	0.35	0.40	103	(2.6)
(11) Propyl Carbamate	0.3	5.6	15.0	0.37			
»	»	4.1	9.7	0.43	0.41	74	4.5
(12) sec.-Butanol	0.3	1.9	4.6	0.41			
»	»	1.9	4.6	0.41	0.44	74	2.7
(13) Methyl Acetate	0.5	3.1	8.0	0.39			
»	0.4	1.9	4.0	0.48	0.49	88	?
(14) Dioxane	0.5	3.7	8.0	0.46			
»	0.3	4.0	7.9	0.51	0.57	60	1.9
(15) n-Propanol	»	3.9	7.4	0.53			
»	»	4.8	7.9	0.61	0.82	132	> 1
(16) Paraldehyde	»	1.9	2.1	0.90			
»	0.15	3.7	5.1	0.73	0.91	114	?
(17) Acetonyl-acetone	0.5	8.3	10.0	0.83			
»	0.3	4.1	4.2	0.98	1.0	32	0.14
(18) Methanol	—	—	—	—			
(19) Ethanol	0.3	4.05	3.8	1.1	1.1	46	0.26
»	0.5	8.9	8.0	1.1			
»	0.3	8.4	7.4	1.1	1.2	89	0.64
(20) Ethyl Carbamate	»	5.5	4.45	1.2			
»	»	3.75	3.8	1.0	1.3	60	?
»	»	19	15	1.3			
(21) iso-Propanol	»	9.5	7.4	1.3	1.4	74	2.2
»	»	4.05	3.15	1.3			
(22) tert.-Butanol	»	10.9	7.4	1.5	1.4	74	2.2
»	»	5.6	4.6	1.2			

Table 1 (continued)

Solute	Molarity	Contraction		RC	MRC	M	k
		I	II				
(23) Acetaldehyde	0.5	15	10	1.5			
»	0.3	6.1	4.2	1.5	1.5	44	0.41
(24) Triethyl Citrate	0.15	9.2	5.1	1.8			
»	0.10	3.1	2.5	1.2	1.5	276	4.4
(25) Glycerol Diethyl Ether	0.3	5.1	3.15	1.6			
»	»	7.0	4.2	1.7	1.6	148	0.84
(26) Ethylene Glycol Monoethyl Ether	»	13.6	9.7	1.4			
»	»	5.25	3.15	1.7	1.6	90	?
(27) Methyl Carbamate	»	8.3	4.45	1.9			
»	»	6.0	3.8	1.6	1.8	75	0.14
(28) Pyramidone	0.15	10.3	5.1	2.0	2.0	231	0.63

January 1950) the results are on the whole fairly consistent. It seems thus a priori plausible to assume that the permeation power of the substances mentioned in the table decreases in about the same order as they are enumerated, i.e., from heavy water to pyramidone.

This supposition can be tested by experiments of a slightly different kind. If a cell is surrounded by a solution of a substance A and this solution is then replaced by an equimolar solution of substance B three alternatives are possible: (1) a reversible elongation, if B permeates more rapidly than A, (2) a reversible contraction, if A permeates more rapidly than B, or (3) no change of length, if B enters the cell with the same rapidity as A is diffusing out. In an experiment of this sort a *Nitella* cell was successively treated with 0.8 molar solutions of methanol, ethanol, and iso-propanol. The course of the experiment may be represented in the following manner:



In this scheme the number above the arrow indicates how many micro-meter scale units the maximum contraction (−) or maximum elongation (+) caused by each change of the medium is. The experiment confirms entirely the earlier conclusion that methanol permeates more rapidly than ethanol or iso-propanol and that ethanol permeates more rapidly than iso-propanol. The order of decreasing permeation power is thus in this experiment also: methanol > ethanol > iso-propanol. It is also seen that the change from the solution of one alcohol to that of another causes but very little change in length as compared with the change from water to an alcohol solution or from such a solution to water. It thus seems that the differences in permeation power between the alcohols tested are considerably smaller than those between the alcohols and water.

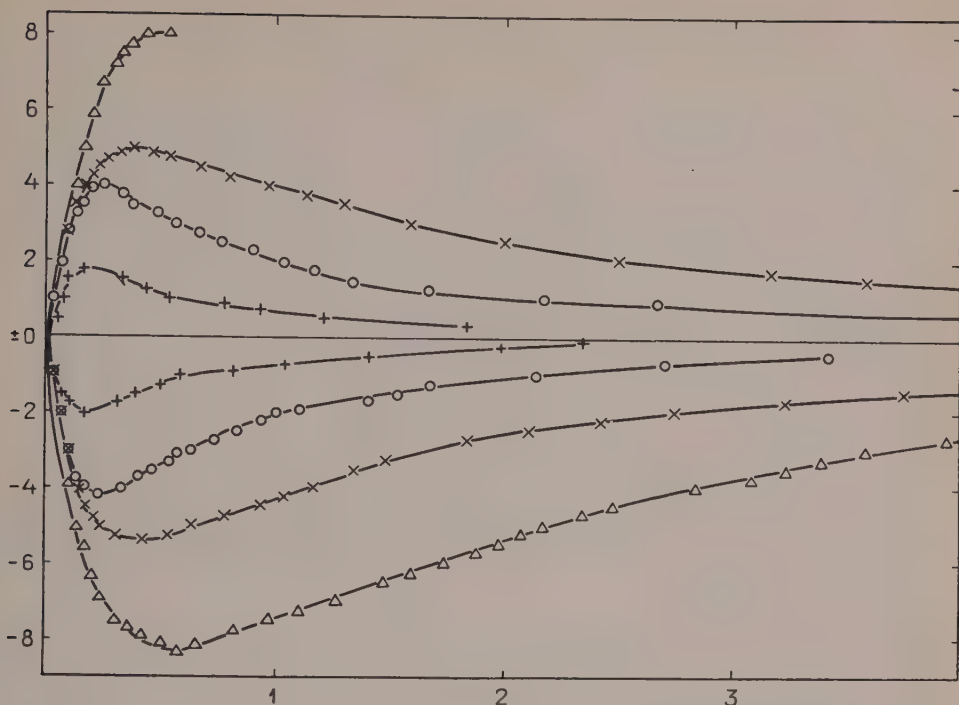


Fig. 4. Contractions of a *Nitella* cell (0.48 mm. thick) under the influence of 0.3 molar solutions of acetone (+), methanol (o), ethyl carbamate (x), and methyl carbamate (Δ) and its subsequent elongations in water. Abscissa: time in minutes. Ordinate: position of the end of the cell on the micrometer scale.

Theoretically a third kind of comparison of the permeation powers should also be possible. As pointed out by Frey-Wyssling (1946) the duration of the contraction, viz. the elongation process, is a function of the permeability of the cell to water on the one hand and to the solute on the other: the more slowly the solute permeates, the later will the flex of the curve be reached. In reality, however, these curves often bend so gradually that it is not easy to establish the exact position of the flex. Thus, in Fig. 3 the difference between the curves are not very clear in this respect although they differ greatly as to their maximum values. The differences as to the duration of the primary contraction or elongation process are, however, much more striking when more slowly penetrating solutes are also tested. Thus Fig. 4 shows that in a certain experiment the flex was reached after 10 sec. with acetone, after 14 sec. with methanol, after 25 sec. with ethyl carbamate, and after 35 sec. with methyl carbamate. It is seen that these data are consistent with the assumption that the order of the substances in Table 1 reflects, at least in general outline, the order of decreasing permeation power.

It should, however, be noted that the mutual consistency of the results arrived at by different methods is, although gratifying in itself, nevertheless

to a considerable degree due to the fact that all these methods have certain weaknesses in common. Especially, it must be emphasized that *the quantity determined in our experiments is not the permeability of the protoplast or of the plasma membrane alone but that of the whole cell including also the cell wall and cell sap*. We have not carried out any experiments designed to reveal how great is the diffusion resistance of the two last-named parts of the cell, but from the investigation of Wartiovaara (1949) it is seen that while the permeability of the whole cells of *Nitella* to methanol, ethanol, n-propanol, and n-butanol at 20° is about 1.1, 1.0, 1.2, and 1.3 cm/hour, respectively, the permeability of the plasma membrane alone is 2.0, 2.0, 3.3, and 4.9 cm/hour, respectively. As is seen from this example and is also theoretically evident, the greater and the more rapidly penetrating the molecules in question, the more the permeability of the plasma membrane alone differs from that of the whole cell.

It seems best to refrain from attempting to calculate any absolute permeation constants for the substances of Table 1, and to content oneself with an only semi-quantitative comparison of the permeation powers as roughly revealed by the table.

In doing so we notice, first of all, that heavy water is by far the most rapidly penetrating of the solutes used in our experiments. Ordinary water evidently penetrates somewhat more rapidly still.

This statement is not contradicted by the findings of Holdheide (1931, p. 247) that *Nitella* cells (species not named), when put into strong solutions of methanol, expand until they burst (plasmolysis). In fact, the *Nitella* cells used by us also sometimes plasmolyzed when treated with methanol solutions, but this was the case only when excessively high concentrations (about 12–14 mol) were used. On the other hand, when the cells were placed, for instance, in an 8 molar solution (about 32 per cent by volume) the cells collapsed visibly in a few seconds. If a few seconds later, they were brought back into water they became in this medium, to begin with, even more turgid than normally. Some of the methanol-treated cells even burst in water. It is thus obvious that *Nitella* cells, in their normal state, are considerably more permeable to water than to methanol and that the reverse state is a quite abnormal one.

Another question is whether the plasma membrane alone is also less permeable to all the substances studied than to water. The observations made scarcely suffice to prove this with certainty. On the other hand it seems very probable that at least substances Nos. 18–28, but fairly probably also, say, Nos. 10–17 must penetrate the plasma membrane with greater difficulty than water, for if this were not the case it would be difficult to explain how, for instance, n-butanol, ethyl acetate, and ethyl ether, whose molecules are distinctly greater than those of, say, methanol, ethanol, and acetone, nevertheless cause only much weaker contractions than these.

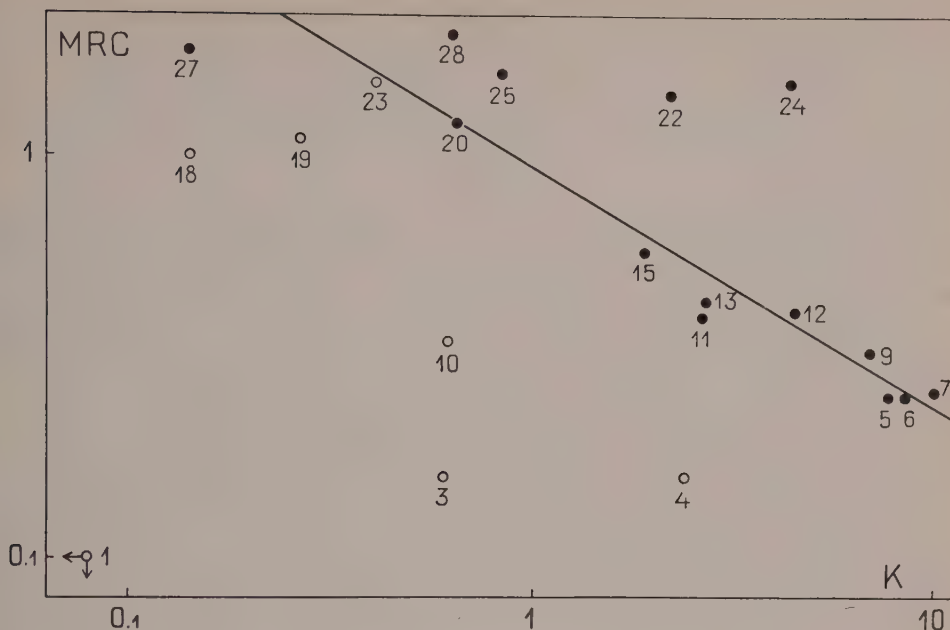


Fig. 5. Relations between contraction power, relative ether solubility, and molecular weight of the substances tabulated in Table 1 as far as their ether solubility is known. ○ mol. weight < 60, ● mol. weight ≥ 60 . Abscissa: distribution coefficient ether/water. Ordinate: mean relative contractions caused by equimolar solutions. The figures of the substances are the same as in Table 1. The arrows from point 1 indicate that the point for heavy water should properly lie outside the diagram.

Fig. 5 gives a general survey of the relations existing between the permeation power on the one hand and the lipid solubility and molecular weight of the solutes on the other. This exposition is admittedly very crude, not only owing to the fact that the lipid solubility is represented simply by the partition coefficient ether/water, but above all because the »relative contractions» caused by the solutes in question were used as a quite rough inverse measure of the permeation power. Nevertheless it is seen that the substances with a molecular weight above 60 are — disregarding two exceptions (triethyl citrate and tert.-butanol) — fairly regularly scattered along a straight line, indicating thus that the relative lipid solubility is the chief factor determining the permeation power of not too small molecules. On the other hand all substances of a molecular weight below 60 are situated below this line, i.e., their permeation power is, so to speak, abnormally great as compared with their ether solubility. Thus some kind of sieve-effect of the plasma membrane seems to be involved here. These results agree

very well, in a general way, with earlier views of the dependence of the permeation power on the lipoid solubility and molecular size. (Cf. Wartiovaara 1949, Collander 1949.)

Summary

The permeability of *Nitella* cells to very rapidly penetrating non-electrolytes can easily be studied semi-quantitatively by watching the changes of length which such cells undergo when treated successively with water and aqueous solutions of the substances to be studied. Table 1 contains an enumeration of the maximum contractions caused by equimolar solutions of 28 such substances. This list at the same time indicates, in a general way, the gradation of permeation power with regard to *Nitella* cells.

It is concluded that the relations between permeation power on the one hand and lipoid solubility (represented by the ether/water partition coefficient) and molecular weight on the other, are in this case about the same as those previously found for other cells and other solutes: for medium-sized molecules the lipoid solubility is the decisive factor while the smallest molecules permeate more rapidly than would be expected in view of their solubility alone.

Thanks are due to my wife for assistance in the experiments, to Dr Veijo Wartiovaara for advice and criticism, and to Professor M. G. Stålfelt (Stockholm) for the original culture of *Nitella*.

References

- Bochsler, A.: Die Wasserpermeabilität des Protoplasmas auf Grund des Fickschen Diffusionsgesetzes. — *Ber. Schweizer Bot. Ges.* 58: 73. 1948.
- Collander, R.: Die Verteilung organischer Verbindungen zwischen Äther und Wasser. — *Acta Chemica Scand.* 3: 717. 1949.
- The permeability of plant protoplasts to small molecules. — *Physiol. Plantar.* 2: 300. 1949.
- and Bärlund, H.: Permeabilitätsstudien an *Chara ceratophylla* II. — *Acta Bot. Fennica* 11: 1. 1933.
- Frey-Wyssling, A.: Zur Wasserpermeabilität des Protoplasmas. — *Experientia* 2: 132. 1946.
- Holdheide, W.: Über einen eigenartigen Fall von Plasmoptyse. — *Biol. Zentralbl.* 50: 705. 1930.
- Über Plasmoptyse bei *Hydrodictyon utriculatum*. — *Planta (Berl.)* 15: 244. 1931.
- Overton, E.: Ueber die allgemeinen osmotischen Eigenschaften der Zelle. — *Vierteljahrsschr. d. Naturforsch. Ges. in Zürich* 44: 88. 1899.
- Saubert, G. G. P.: The influence of alcohols on the protoplasmic membrane and colloid models. — *Rec. Trav. Bot. Néerl.* 34: 709. 1937.

- Wartiovaara, V.: The permeability of *Tolypellopsis* cells for heavy water and methyl alcohol. — *Acta Bot. Fenn.* 34:1. 1944.
- The permeability of the plasma membranes of *Nitella* to normal primary alcohols at low and intermediate temperatures. — *Physiol. Plantar.* 2:184. 1949.
- Zehetner, H.: Untersuchungen über die Alkoholpermeabilität des Protoplasmas. — *Jahrb. wiss. Bot.* 80:505. 1943.

The Growth-Inhibiting Action of Thiophenoxy Acetic Acids

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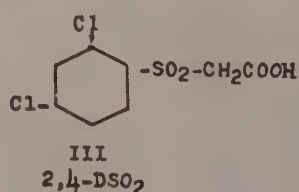
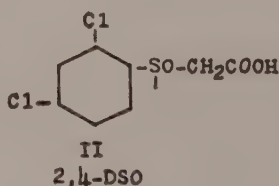
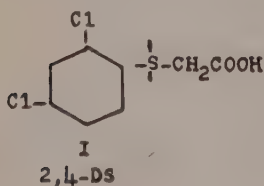
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Introduction

Erdtman (6) has recently drawn attention to the fact that in anaesthetics of the xylocain type the $C=C$ double bond may be replaced by a $-C=C-$ group without loss of activity. Both N-dimethylaminoacet-*o*-toluidine and α -dimethylaminomethyl-indole are active as local anaesthetics (5).

Erdtman further points out that similar structural features occur in several growth substances of the auxin type. According to the rules found for the structural requirements of growth substances (cf. Thimann, 7) the ring must contain a double bond adjacent to the side chain. This is fulfilled by the pyrrole ring in β -indolylacetic acid. For the somewhat modified requirements of the hemiauxins see Went (9). Erdtman suggests the possibility that the lone pairs of electrons on the ether oxygen atom of the phenoxy acids may exert the same influence as the π -electrons of the indole $-CH=CH-$ double bond.

A first attempt to test this hypothesis was made by Erdtman and Nilsson (6) who prepared the sulphur analogue of 2,4-dichlorophenoxyacetic acid (I), here termed 2,4-DS. By successive oxidation the corresponding sulfoxide (II), 2,4-DSO, and sulphone (III), 2,4-DSO₂, were obtained:



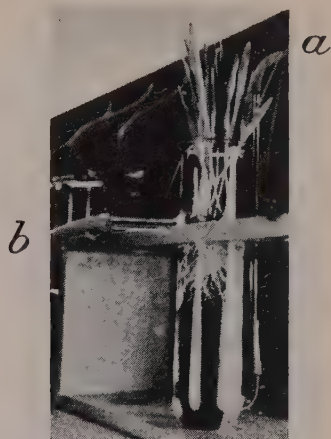


Fig. 1. Culture vessel with plants. a, Inlet; b, Outlet for the solution.

The lone pairs of electrons of the sulphur atoms are less available for association and conjugation than those of the oxygen atoms, and the lone pair of electrons left on the sulphur atom of the sulphoxide group should be still less available. In the sulphones there are no lone pairs of electrons. — The lone pairs of electrons are indicated by — in the formulae I and II. — Hence it was suggested that the activity of 2,4-dichlorothiophenoxyacetic acid (I), the sulphoxide (II), and the sulphone (III) might decrease in this order.

In the present investigation these substances have been tested as to their growth-inhibiting action by a detailed study of their influence on the elongation of roots of oats.

Methods

Seeds of Weibull's Bambu oats were soaked for 24 hours in water, the awns removed and the seeds germinated for 60 hours in Petri dishes. They were then transferred to cork disks and placed for 24 hours in a solution of .0010 mol $\text{Ca}(\text{NO}_3)_2$ and .0005 mol K_2SO_4 in distilled water free from heavy metals at 20°C and constant artificial light. Each disk with 10 plants was then transferred to a test vessel (fig. 1) containing about 120 ml. of the same solution with or without additions of the acids. These were supplied in concentrations from 10^{-8} to 10^{-4} mol. Well-aerated stock solutions flowed from 2-litre flasks continuously over the roots during the whole time of experiment at a rate of about 750 ml. per 24 hours, thus maintaining a constant composition of the solution bathing the roots. The rate of flow was regulated by capillaries. Four treatments were run simultaneously, each

*a**b**c**d*

Fig. 2. General view of the apparatus for tests with flowing solutions. a, stock flasks with aeration tubings; b, siphons for the solutions with glass capillaries regulating the rate of flow; c, Culture vessels for the plants; d, outlets for the solutions to drainage flasks.

with three replicates. The entire apparatus is shown in fig. 2. The stock flasks were refilled daily with freshly prepared solutions without interruption of the tests. The pH of the solutions varied between 5.4 and 5.9. All experiments were carried out in darkness at a temperature of 19.5° to 20.5° C.

At the start of the experiment all roots were cut off except one of the first lateral, adventitious roots on each seedling. The lengths of these remaining single roots were recorded. After one, two, and three days ten plants of each treatment were harvested and the root lengths measured. In most series the roots were subjected to a microscopical investigation. The lengths of the epidermal cells were measured at the end of the zone of elongation, each series of measurements included about 100 cells. For the critical concentrations the tests were repeated up to four times.

The mean errors of the root lengths amounted to less than 10 per cent for control plants and those from less toxic solutions; somewhat higher figures were obtained with more toxic concentrations. The mean errors of the cell lengths varied between 2 and 4 per cent. The ratio, *increase in root length : cell length*, was also computed giving a figure of the cell multiplication (cell number) in the longitudinal direction of the root (cf. Burström, 2).

Results

The control series without added acids were repeated five times with fairly good agreement between the series (table 1). One striking feature is that the growth rate increases from the first to the second day and then decreases again. The same was found in the series with weak or moderate inhibitory action of the acids. The increasing growth rate is a common phenomenon with seedlings, the cause of which has not been investigated. The ensuing decrease, on the other hand, may depend upon the fact that the tests were carried out in darkness and may be connected with incipient starvation of some growth factor. Thus, it is obviously necessary to pay attention to the time course of the action of the acids.

The general effect of the acids on the root elongation is shown by fig. 3, illustrating the time course of the increase in length of the roots.

A point of interest is that the variations between the series in cell lengths and cell multiplication are smaller than those of the total root lengths, which justifies treating these separately as two primary properties.

This is also shown by the way in which 2,4-D affects the roots (tables 2 and 3). Table 2 gives the average figures for cell lengths and rates of multiplication of the main part of the experimental material, in table 3 the effects of 2,4-D and 2,4-DS have been computed in relation to the controls. This table shows that the cell lengths of the 2,4-D treated plants fluctuate around average values of 56 per cent for 10^{-7} mol and 31 per cent for 10^{-6} mol, which means a reduction in the cell elongation by 44 and 69 per cent respectively. The cell multiplication, on the contrary, decreases steadily from the first to the third day in the higher concentration but is very little affected at a lower addition of the acid. That would imply a rapid and rather constant inhibition of the elongation to a level characteristic of the prevailing concentration of 2,4-D in the external medium. Since the effect on the multi-

Table 1. *Data of control roots from solutions without added acids.* Figures given for three consecutive days. Root length denotes increase in length per day. Cell number is computed as the ratio increase in root length : cell length.

Exp. nr	Root length mm			Cell length μ			Cell number		
	Day			Day			Day		
	1	2	3	1	2	3	1	2	3
I	9.5	30.4	—	—	—	—	—	—	—
II	11.3	31.7	20.1	268	395	399	42	80	50
III	10.6	28.5	29.4	265	464	412	40	61	71
IV	8.8	24.9	20.4	206	355	349	43	70	59
V	13.1	36.4	—	286	427	—	46	85	—
Average ...	10.7	30.4	23.3	256	411	386	43	74	60

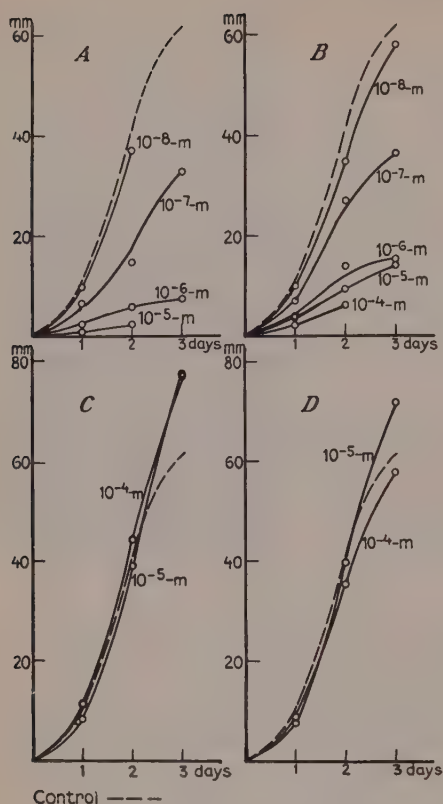


Fig. 3. The elongation of roots for three days kept in nutrient solutions with additions of:

A 2,4-D	C 2,4-DSO
B 2,4-DS	D 2,4-DSO ₂ .

The figures denote concentrations of the acids in mols.

plication is cumulative, it may be that it increases with an accumulation of the acid in the root or by a penetration into the meristem. Audus (1) has stated that the inhibition of the root elongation may be regulated by the rate of entry of 2,4-D; elongation is then expressed only as the net increase in length of the root, so that a detailed comparison with our results is impossible. His material, pea and cress, seems to be surprisingly insensitive to the acids, however, the lowest concentration supplied, about $5 \cdot 10^{-7}$ mol and $5 \cdot 10^{-5}$ mol, still allowing a growth of the pea roots.

In 10^{-8} mol the effect was not statistically significant and in 10^{-5} mol no growth was obtained; the roots died. In 10^{-6} mol the root tips showed swellings in the elongation zone and excessive formation of root hairs, the usual consequences of an inhibition of the longitudinal stretching of the cells.

The assumption that the primary action of 2,4-D consists in a decrease in the rate of the cell elongation and that the change in rate of cell multiplication may be regarded as a secondary phenomenon gains support from

Table 2. *Lengths of epidermal cells and cell number in longitudinal direction of roots treated with 2,4-D-derivatives. Values given after 1, 2, and 3 days.*

Substance	Conc. mol	Cell length μ			Cell number		
		1 day	2 day	3 day	1 day	2 day	3 day
None	—	256 \pm 6	411 \pm 7	386 \pm 8	43	74	60
2,4-D	10 ⁻⁷	163 \pm 5	186 \pm 6	228 \pm 7	38	52	43
	10 ⁻⁶	106 \pm 4	89 \pm 4	120 \pm 4	25	33	12
2,4-DS	10 ⁻⁷	186 \pm 5	358 \pm 8	218 \pm 9	37	56	51
	10 ⁻⁶	116 \pm 4	219 \pm 7	164 \pm 7	37	46	12
	10 ⁻⁵	133 \pm 7	108 \pm 5	(143 \pm 6)	20	39	—
2,4-DSO	10 ⁻⁵	327 \pm 10	475 \pm 15	469 \pm 18	32	64	81
	10 ⁻⁴	294 \pm 8	525 \pm 16	517 \pm 15	34	63	66
2,4-DSO ₂	10 ⁻⁵	225 \pm 9	525 \pm 16	468 \pm 17	29	72	58
	10 ⁻⁴	214 \pm 7	378 \pm 10	458 \pm 14	36	52	76

Table 3. *The relative effects of 2,4-D and 2,4-DS on cell elongation and cell multiplication. Cell lengths and cell numbers of controls=100.*

Substance and concentration	Elongation			Multiplication		
	day			day		
	1	2	3	1	2	3
2,4-D						
10 ⁻⁷	64	46	58	88	70	72
10 ⁻⁶	41	22	31	57	44	19
2,4 DS						
10 ⁻⁷	73	87	72	88	76	85
10 ⁻⁶	45	53	42	87	61	19

a comparison of 2,4-D with 2,4-DS (table 3). The results are very similar to those with 2,4-D, the elongation is less inhibited, however, only about 23 and 53 per cent in 10⁻⁷ and 10⁻⁶ mol respectively. Rather constant figures were obtained for the three days of experiment. The effect on the cell multiplication, on the contrary, is very much the same as with 2,4-D, a very slight or no effect at 10⁻⁷ mol, and a declining rate of cell divisions in the higher concentration. In 10⁻⁵ mol solution of 2,4-DS the roots did not die, but the elongation was too slight to be measured with any degree of accurateness.

A graphic interpolation has revealed that 2,4-D is about three times as active in inhibiting the cell elongation as 2,4-DS. This difference is very small as compared with the pronounced difference between these compounds on one hand, and the following two on the other (table 2).

The introduction of an oxygen atom on the sulphur apparently abolishes the physiological activity. No consistent inhibition of the root growth was observed in any concentration of 2,4-DSO and 2,4-DSO₂. In 10⁻⁵ mol there is a statistically significant increase in the cell length of up to 30 per cent,

whereas the cell number is lower than that of the control plants for the first day and rises to a higher figure for the third day. For all three days together the cell number of the control roots amounted to 177, for 10^{-5} 2,4-DSO to 176 and for 10^{-4} mol to 163. It may be assumed that this shift between the three days is of secondary importance and that the rate of cell multiplication is nearly unaffected by this acid even in the high concentration of 10^{-4} mol.

The action of 2,4-DSO₂ is very similar, although the results due to larger variation are only partly statistically significant. The change in the time course of the cell divisions with a lower figure for the second and a higher figure for the third day is undoubtedly significant, and has been especially corroborated in repeated experiments. The cause is unknown, but a comparison may be made with the protecting action of herbicides, as studied by Currier (4).

In any case, the difference between 2,4-D and 2,4-DS on one hand and 2,4-DSO together with 2,4-DSO₂ on the other is striking. The growth inhibition has disappeared so completely in the second group of acids that it is not even possible to compute a reliable figure for the activity relative to 2,4-D. This acid must be at least 10,000 times more active, if the sulphoxide and sulphone acids exert any activity at all.

Discussion

The marked difference between the two pairs of acids is illustrated in fig. 4, which shows the data for the second day of cell lengths, cell numbers, and total root lengths corresponding to the concentrations of the acids. The two groups of values fall so far apart from each other that there seems to be some kind of all-or-nothing effect in this series of derivatives. Such a conclusion is hardly necessary, however. — Roots of the same length can be comprised of either a higher number of smaller cells, or a lower number of more elongated cells; several examples of the relative independence of these two properties have been given in earlier papers (cf. 3). If there is only one kind of physiological activity of the studied acids, a certain root length must correspond to a given relation between cell length and cell number, irrespective of whether it is caused by a higher concentration of a less active or a lower concentration of a highly active compound.

In order to test whether this holds true in the present case fig. 5 has been drawn, where cell lengths and numbers have been plotted against root lengths for all four acids in the different concentrations employed in the experiments. It appears that the values for cell length and cell number

Fig. 4. The general trend in the action of 2,4-D-derivatives on cell elongation, cell multiplication, and total root length. The graph refers to the second day of experiment. The figures for control roots = 100.

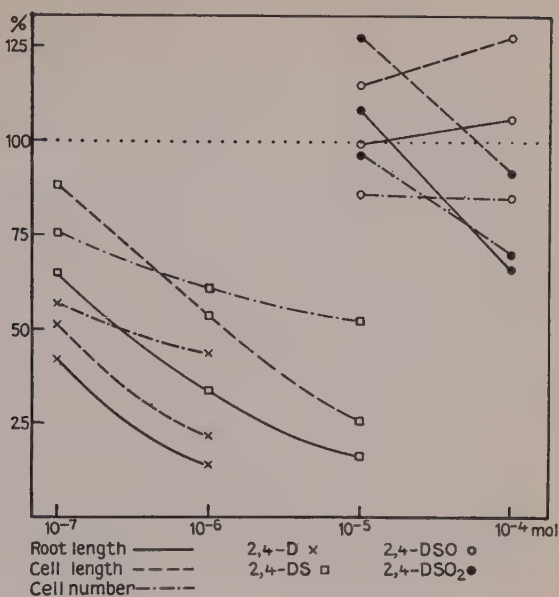
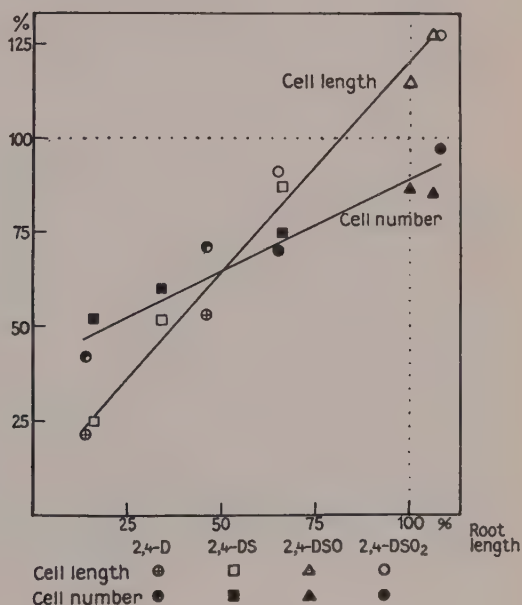


Fig. 5. The relation between root length, cell length, and cell multiplication. The abscissa represents root length in per cent of the control, the ordinate represents cell length and cell number. Data from the second day and all concentrations of the tested acids.



fall smoothly around two straight lines. The graph showing the cell length declines more rapidly than that for the cell number in accordance with the previously discussed more obvious influence of the acids on the elongation.

It must especially be remarked that the figures for the increase in cell length observed with the sulfoxide do not fall outside the regular line showing the action on the cell elongation. Thus it is possible, although far from certain, that the acids exhibit the same action on the cell elongation but only with different strengths.

Another point of importance is the dissociation of the acids. They are all very strong as compared with most other growth active substances. The K_s of 2,4-D amounts to $5.2 \cdot 10^{-4}$ (Audus, 1 a), that of phenoxyacetic acid to $7.5 \cdot 10^{-4}$. The introduction of two Cl very little affects the dissociation. Constants for the di-halogenated sulphur derivatives are lacking, but according to available data of hand-books K_s of phenylthioglycolic acid amounts to $3.0 \cdot 10^{-4}$; the thiocompounds thus differing very little from the oxyacids. The non-halogenated sulphony- and sulphone acetic acids have K_s of $2.2 \cdot 10^{-3}$ and $4.5 \cdot 10^{-3}$ respectively. The constants for the di-chlor-derivatives ought to lie near these values. Even for 2,4-D the concentration of undissociated acid amounts to less than .5 per cent at pH 5.5, and for the sulphony and sulphone acetic acids to about .15 and .07 per cent respectively. The actual concentrations of undissociated acids are in any case extremely low in the physiologically active solutions, so that it is hardly possible to assume that the acids only act in undissociated form, and that a further increase in dissociation could explain the differences between the acids. Furthermore, the thio-acid is 2 or 3 times weaker than the oxy-acid, and its activity is only one third.

The inactivity of the acids II and III obtained on oxidation of the thiophenoxy acid (I) is in agreement with Erdtman's (6) hypothesis that one structural requirement of the active substances is the availability of electrons at a suitable distance from the carboxyl group. This question is further studied by Erdtman and his coworkers, who are engaged in the synthesis of new compounds of this type.

Reference should also be made to the opinion advanced by Veldstra (8) that the activity depends upon the ability of the sidechain to attain a position more or less perpendicular to the plane of the ring. According to a private communication by Dr. Erdtman the thiophenoxy acids I—III, however, should be closely similar to the phenoxy acids in this respect. Therefore, the fulfillment of this requirement does not explain the decreasing activity.

Summary

The growth activity of 2,4-dichlorophenoxyacetic acid (2,4-D) and its sulphur derivatives 2,4-dichlorothiophenoxyacetic acid (2,4-DS), 2,4-dichlor-

phenylsulphoxyacetic (2,4-DSO) and 2,4-dichlorophenylsulphoneacetic acid (2,4-DSO₂) has been studied on roots of oats. The tests were carried out with constantly flowing solutions and by a microscopical investigation of the roots.

The primary action of 2,4-D and 2,4-DS is a retardation of the cell elongation amounting to about 50 % in 10^{-7} mol 2,4-D; by prolonged treatment also the cell multiplication will decrease. In the first respect 2,4-D is about three times as active as 2,4-DS. No significant growth inhibition was obtained with 2,4-DSO and 2,4-DSO₂, not even in 10^{-4} mol solutions, on the contrary, a significant increase in the cell elongation was found, especially with 2,4-DSO.

The results are briefly discussed in terms of the hypotheses of Erdtman and Veldstra.

References

1. Audus, L. J.: Studies on the phytostatic action of 2,4-dichlorophenoxyacetic acid and coumarin. — *New Phyt.* 47: 196. 1948.
- 1 a. — Studies on the pH-relationships of root growth and its inhibition by 2,4-dichlorophenoxyacetic acid and coumarin. — *Ibid.* 48: 97. 1948.
2. Burström, H.: On formative effects of carbohydrates on root growth. — *Bot. Notiser* 1941: 310. 1941.
3. — A preliminary study on mineral nutrition and cell elongation of roots. — *Fys. Sällsk. Lund Förh.* 17: 1. 1947.
4. Currier, H. B.: Responses of plant cells to herbicides. — *Plant Phys.* 24: 601. 1949.
5. Erdtman, H. & Löfgren, L.: Über eine neue Gruppe von lokalanästhetisch wirksamen Verbindungen. — *Svensk Kem. Tidskrift* 49: 163. 1937.
6. Erdtman, H. & Nilsson, G.: Synthetic plant hormones. I. Sulphur analogues of some phenoxy acetic acids. *Acta Chem. Scand.* 3: 899. 1949.
7. Thimann, K. V.: Plant hormones. — *The hormones* 1: 5. New York 1948.
8. Veldstra, H.: Researches On Plant Growth Substances. V. Relations between chemical structure and physiological activity I. — *Enzymologia* 11: 97. 1944.
9. Went, F. W.: Phytohormones: Structure and physiological activity. II. — *Arch. Bioch.* 20: 131. 1949.

The Influence of Lysine on the Growth of Arginineless Mutants of *Ophiostoma multiannulatum*

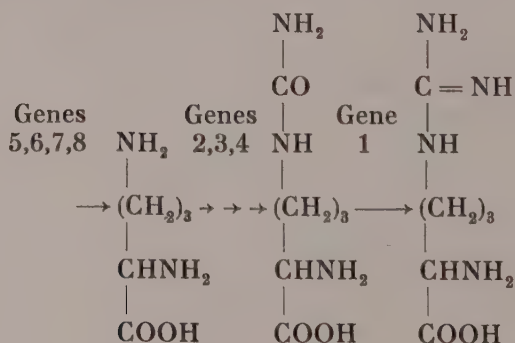
By

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(Received March 7, 1950)

The studies of the metabolism of arginineless mutants of *Neurospora* (14, 15), *Penicillium* (1), *Escherichia coli* (16) and of lactic acid bacteria (17) have shown that the same types of mutants occur in the different species (16). Arginine appears to be formed from ornithine according to the same general pattern as in the Krebs-Henseleit (12, 13) ornithine cycle that occurs in mammalian tissue. The results of the work on arginineless *Neurospora* mutants by Srb and Horowitz (14, 15) are summarized in the following series of reactions:



So far only one mutant has been found that can utilize arginine but not citrulline or ornithine. Three genetically different mutants have been isolated that can use both arginine and citrulline but not ornithine, i.e. the formation

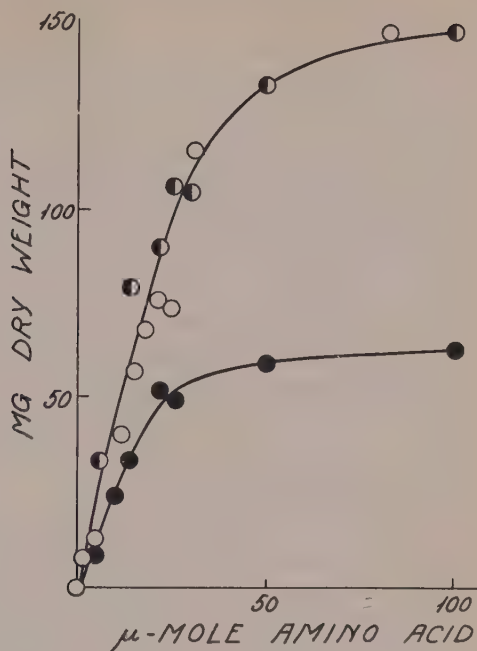


Fig. 1. Growth curves for *Ophiostoma* mutant 1442 with L-arginine (open circles), L-citrulline (half filled circles), and L-ornithine (filled circles).

of citrulline from ornithine can be blocked in at least three different ways presumably by the inability of the mutants to accomplish three different chemical reactions necessary for the formation of citrulline from ornithine. In this case the block in the genetically different mutants have not yet been connected with certain intermediate reactions or with the lack of specific enzymes. In the remaining four mutants the block is earlier than ornithine.

In the present investigation 81 arginineless mutants of *Ophiostoma multiannulatum* have been investigated so far. These mutants are part of the numerous arginineless mutants that have been isolated by Dr N. Fries (6, 7, 8) in Upsala during the last several years. They have not yet been investigated from a genetical point of view and many of them certainly carry identical mutant genes.

These mutants did not grow at all on the basal medium at 25° but all grew well when 15 micromoles L-arginine was added.

15 of the 81 mutants could only use L-arginine but not L-citrulline or L-ornithine, 42 could use arginine and citrulline but not ornithine and the remaining 24 could use all three amino acids.

A typical set of growth curves are plotted in figure 1 showing the growth response of the arginineless mutant 1442. L-arginine and L-citrulline gave practically identical curves whereas L-ornithine leveled off at a much lower

level. This was the case also in other mutants of the same type, whereas in *Neurospora* the difference was much less pronounced (14).

The investigation of the last group as to their utilization of proline and glutamic acid (1) is not yet complete and will be reported later.

The results obtained thus indicate that the arginine formation in *Ophiostoma* takes place according to the same general pattern as in other organisms investigated and that the same type of reactions are blocked by mutations in *Ophiostoma* as in *Neurospora*, *Penicillium*, and *E. coli*.

Cohen and Grisolia (2) have found that carbamyl-L-glumatic acid might function as an intermediate in the synthesis of citrulline from ornithine in rat liver homogenates. This compound has therefore been tested on the arginineless *Ophiostoma* mutants as the block in the arginine synthesis in some mutants might be caused by inability to synthesise this compound. None of the 81 mutants tested so far have been influenced by the addition of carbamyl-L-glumatic acid.

Srb (15) recently also tested carbamyl-L-glumatic acid on three genetically different arginineless *Neurospora* mutants (30820) that could use citrulline but not ornithine, in which thus the mutation had given rise to an inability to convert ornithine to citrulline. However, as in the present investigation no effect was observed on these or other types of arginineless mutants.

If carbamyl-glutamic acid is an intermediate in the citrulline synthesis in these organisms is therefore still unknown.

A number of facts pointing to the interrelation of the metabolism of arginine and lysine are known. Only to mention the evidence in *Neurospora*, Houlahan and Mitchell (11) reported that an arginineless mutant of *Neurospora* (36703) that could utilize arginine but neither citrulline nor ornithine was inhibited by lysine when the molar ratio of lysine to arginine was 3.5 or higher.

Furthermore Doermann (4) found that three genetically different lysine mutants all were strongly inhibited by addition of arginine already when the molar ratio of arginine to lysine was about 1. A fourth mutant behaved in the same way (5). The other common amino acids did not have this effect.

Another case of lysine-arginine interaction was observed by Houlahan and Mitchell (10, 11) in connection with the study of a suppressor in certain pyrimidineless *Neurospora* mutants. Some pyrimidineless mutants carrying certain mutated genes did not grow without the addition of pyrimidines to the medium. When the suppressor was also present, the mold had partially recovered the ability to grow in the absence of pyrimidines but only showed half maximum growth on the basal medium. However, addition of small amounts of arginine to this medium completely inhibited the effect of the suppressor so that no growth was possible in the absence

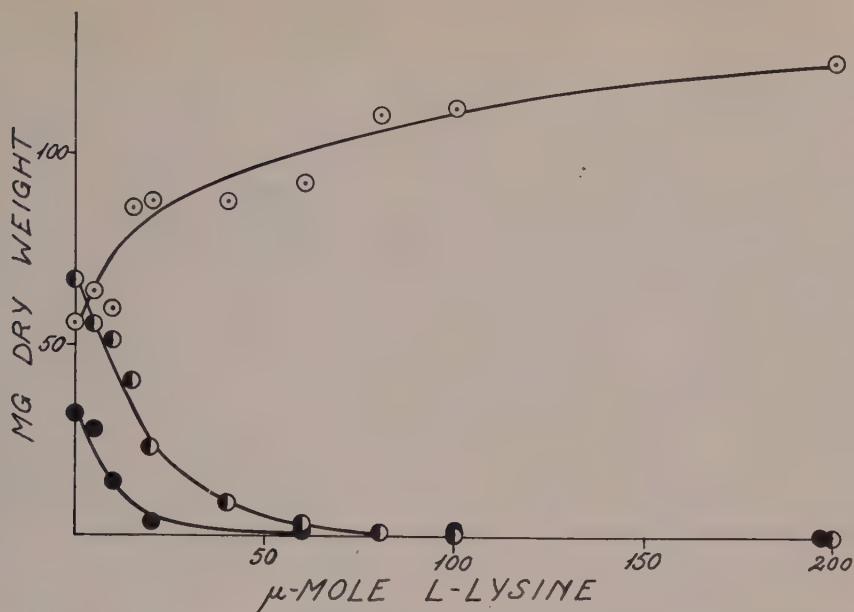


Fig. 2. Influence of L-lysine on the growth of mutant 1442 in the presence of 20 micromoles of respectively L-arginine (open circles), L-citrulline (half filled circles), and L-ornithine (filled circles).

of pyrimidines. Citrulline and ornithine had a similar but much weaker effect.

This growth inhibition with arginine could be overcome not only by addition of pyrimidines but also by lysine.

Horowitz and Srb (9) observed that canavanine strongly inhibited the growth of some wild strains of *Neurospora*. The inhibition was reversed by arginine but in some strains also by lysine.

Lysineless *Ophiostoma* mutants, that are under investigation at present in this institute, are also inhibited by arginine although at somewhat higher ratios of arginine to lysine than reported by Doermann (4) for lysineless *Neurospora* mutants.

However, contrary to the reported inhibition by lysine of arginineless *Neurospora* mutants growing on arginine the corresponding *Ophiostoma* mutants are stimulated to a certain degree by the presence of lysine in addition to arginine in the medium.

A typical case is illustrated in figure 2 showing the influence of increasing amounts of lysine on the growth of an arginineless mutant (1442) in the presence of 20 micromoles arginine. A similar stimulation has been observed

with mutants of all three types, i.e. utilizing only arginine, arginine and citrulline or arginine, citrulline and ornithine when growing on a limited amount of arginine.

However, when the arginineless *Ophiostoma* mutants of the last two types were grown on citrulline respectively citrulline or ornithine, addition of lysine had a very pronounced inhibiting effect that is also exemplified in figure 2. In the presence of 20 micromoles citrulline already about 15 micromoles lysine reduced the growth to half, i.e. at a ratio of lysine to citrulline of 0.75. In the presence of 20 micromoles of ornithine the growth response was reduced fifty per cent by the addition of only 10 micromoles lysine. In both cases 2—3 times the amount of lysine that caused half-inhibition completely inhibited growth. When the amount of citrulline and ornithine was doubled (40 micromoles), the amount of lysine required for half inhibition was also doubled, i.e. in this region it is the ratio between the concentration of lysine and citrulline or ornithine that is determining the inhibition, as would be expected for a competitive analogue-metabolite growth inhibition.

It might indicate that free lysine could compete with ornithine when the latter is an essential metabolite that has to be taken up from the medium. However, the competitive inhibition by lysine of the utilization of citrulline in arginineless mutants that cannot use ornithine must have another explanation. The inhibition must in the latter case be connected with some reaction directly or indirectly connected with the transformation of citrulline to arginine. A wild strain was not inhibited by lysine.

That the observed lysine inhibition is concerned with the synthesis of arginine is in accord with the fact that no lysine inhibition occurs when arginine is present in the medium. The stimulating effect of lysine in this case might be a sparing action of some kind.

Some interesting possibilities for the interaction of lysine, ornithine and arginine can be speculated upon, should the suggestion (3) that homo-arginine occurs as a metabolite in nature be confirmed.

More work is needed to clarify the mechanism underlying the observations recorded in this paper. It would be of interest to know if the observed lysine inhibition also occurs in arginineless mutants of other species.

Methods

The mutants have been kept on agar slant tubes of Fries' »complete agar medium». After inoculation the tubes are kept at 25° until the mutant has grown out. They are then stored at +4° for at most 3 months before reinoculation.

Composition of medium:

15 g agar
25 g malt extract
2.5 g casein hydrolysate
2.5 g yeast extract
1000 ml dist. water.

The growth experiments have been made in 125 ml Erlenmeyer flasks containing 25 ml of the following »modified medium 3» of Fries (l.c.) that support the growth of the wild strains of *Ophiostoma multiannulatum*:

20 g glucose
5 g ammonium tartrate
1 g KH_2PO_4
0.5 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$
0.1 g NaCl
0.1 g CaCl_2
0.00443 g $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$
0.00405 g $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$
0.004 g $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$

1 ml of a stock solution containing 40 γ vitamin B1 and 40 γ B6 per ml

Distilled water to 1000 ml.

25 ml of this medium was added to each flask. The compounds to be tested (that were not destroyed by the heat sterilization) were added and the flasks sterilized with steam at 120° for 20 minutes. The pH of the sterilized media was 5.5—5.6. The flasks were then inoculated from the agar slants and incubated at 25° for 3 weeks unless stated otherwise.

The mycelia were collected by centrifugation in weighed centrifuge tubes, washed twice with distilled water and dried to constant weight at 105°.

Summary

1. A number of arginineless mutants of *Ophiostoma multiannulatum* have been investigated. The results indicate that arginine is formed from ornithine via citrulline as found in other species.

2. Lysine strongly inhibited the growth of arginineless mutants on ornithine or citrulline but stimulated the growth in the presence of arginine.

3. Some of the implications of these results have been discussed.

We are greatly indebted to Dr Nils Fries, Upsala, for placing the mutants at our disposal and for valuable help.

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References

1. Bonner, D.: Production of biochemical mutations in penicillium. — Amer. J. Botany 33: 788. 1946.

2. Cohen, P. P. & Grisolia, S.: The intermediate rôle of carbamyl-L-glutamic acid in citrulline synthesis. — *J. Biol. Chem.* 174:389. 1948.
3. Dent, C. E.: A study of the behaviour of some sixty amino-acids and other ninhydrine-reacting substances in phenyl-collidine filter paper chromatograms, with notes as to the occurrence of some of them in biological fluids. — *Biochem. J.* 43:169. 1948.
4. Doermann, A. H.: A lysineless mutant of *Neurospora* and its inhibition by arginine. — *Arch. Biochem.* 5:373. 1949.
5. — Private communication. 1949.
6. Fries, N.: X-ray induced mutations in the physiology of *Ophiostoma*. — *Nature* 155:757. 1945.
7. — Experiments with different methods of isolating physiological mutations of filamentous fungi. — *Nature* 159:199. 1947.
8. — Spontaneous physiological mutations in *Ophiostoma*. — *Hereditas* 34:338. 1948.
9. Horowitz, N. H. & Srb, A. M.: Growth inhibition of *Neurospora* by canavanine, and its reversal. — *J. Biol. Chem.* 174:371. 1948.
10. Houlahan, M. B. & Mitchell, H. K.: A suppressor in *Neurospora* and its use as evidence for allelism. — *Proc. Nat. Acad. Sci.* 33:223. 1947.
11. —, — Evidence for an interrelation in the metabolism of lysine, arginine and pyrimidines in *Neurospora*. — *Proc. Nat. Acad. Sci.* 34:465. 1948.
12. Krebs, H. A. & Henseleit, K.: Untersuchungen über die Harnstoffbildung im Tierkörper. — *Z. physiol. Chem.* 210:33. 1932.
13. Ratner, S. & Pappas, A.: Enzymatic mechanism of arginine synthesis. — *Federation Proc.* 8:241. 1949.
14. Srb, A. M. & Horowitz, N. H.: The ornithine cycle in *Neurospora* and its genetic control. — *J. Biol. Chem.* 154:129. 1944.
15. Srb, A. M.: Lack of effect of carbamyl-L-glutamic acid on the growth of certain arginineless mutants of *Neurospora*. — *Arch. Biochem.* 23:495. 1949.
16. Tatum, E. L.: Induced biochemical mutations in bacteria. — *Cold Spring Harbor Symposia* 11:278. 1946.
17. Volcani, B. E. & Snell, E. E.: The effects of canavanine, arginine, and related compounds on the growth of bacteria. — *J. Biol. Chem.* 174:893. 1948.

Influence of Cellulolytic Enzymes from Hymenomycetes on Cellulose Preparations of Different Crystallinity

By

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Since several years (Kellerman and Mc Beth 1912), bacteria and fungi have been isolated and qualitatively tested for their ability to break down cellulose on agar plates containing a medium with precipitated cellulose. In the opaque agar, the production of extracellular cellulose-decomposing enzymes, which transform insoluble cellulose to soluble sugars, will bring about cleared, easily visible zones around the fungal or bacterial colonies. In this manner the present author tested the cellulolytic power of certain species of the hymenomycete genus *Tricholoma*, in some cases with positive results (Fig. 1).

Although a wide range of fungi, by means of this or other methods, has been found capable of attacking cellulose, the cellulolytic enzyme activity in cell-free solutions has been investigated only in the case of a few moulds and wood-rotting fungi. Grassmann and collaborators (1931, 1933 a and b), Freudenberg and Ploetz (1939) examined the enzyme mixture obtained from *Aspergillus oryzae* and some other moulds. Reese (1946) studied the cell-free filtrate of *Aspergillus fumigatus*; Saunders *et al.* (1948) that of *Myrothecium verrucaria*. Eight wood-destroying Polypores, cultivated on malt extract, were investigated with respect to their extracellular and intracellular enzymes by Bose and associates (1937, 1939, the later publication also cites the older literature). The paper of Ploetz (1939) deals with enzyme mixtures of press juice from *Merulius lacrimans* cultures.

By continued, physiological comparisons between mycorrhiza-forming and non-mycorrhiza-forming *Tricholoma* species (Norkrans 1944, 1949), the author found need for a sensitive, rapid method of testing for cellulose-decomposition.

Generally, the cellulolytic enzyme activity has been expressed in terms of the amount of reducing sugars formed by the enzymic hydrolysis of the substrate. Different sources of cellulose have been used as substrate, for instance, precipitated cellulose, filter paper, wood cellulose, de-waxed cotton and, often, lichenin, the so-called »reserve cellulose». — Sometimes the decrease in tensile strength of fiber celluloses by enzyme activity has been determined. — Of the substrates mentioned, all the native celluloses require a long time for decomposition, while the lichenin is the most easily-attacked. Meyer *et al.* (1947), however, have shown that lichenin and cellulose differ considerably more in their constitution than has been suggested earlier; the glucose units of the lichenin are linked only to about 73 per cent in 1,4-position, the remaining 27 per cent in 1,3, giving an irregular chain. Levinson and Reese (1949) have measured the cellulolytic activity, viscometrically, with carboxymethyl cellulose as substrate, first proposed for microbiological works by Freeman *et al.* (1948). Unfortunately this substrate also diverges chemically rather markedly from the native cellulose.

Regarding these facts, it occurred to the author that it would be best to try to obtain cell-free solutions from the *Tricholoma*-species and to determine, *photometrically*, their cellulolytic enzyme activity on a precipitated cellulose.

Material and Methods

Testorganisms. Pure cultures of *Tricholoma nudum* (Bull. ex Fr.) (= *Rhodopaxillus nudus* R. Maire) and *Tr. fumosum* (Fr.) (non Pers.) isolated by the author, were used. Two wood destroying fungi, namely *Polyporus annosus* Fr. and *Coniophora puteana* (Schum. ex Fr.) Karst., were also studied, in order to compare their cellulolytic activity with that of the soil-inhabiting *Tricholoma* species.

A culture of *P. annosus*, belonging to the stock culture collection of Prof. Elias Melin, Uppsala, was courteously placed at the author's disposal while *C. puteana* was kindly supplied by Dr. Stig O. Pehrson, Swedish Forest Products Research Laboratory, Stockholm.

Nutrient solution. The nutrient solution had the following composition: cellulose (swedish filter paper, Munktell n:r 3, chopped and washed by shaking in distilled water) 5.0 g, $\text{NH}_4\text{H}_2\text{PO}_4$ 2.0 g, KH_2PO_4 0.6 g, K_2HPO_4 0.4 g, $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ 0.5 g, ferrictrate (dissolved in citric acid) 5 mg, $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ 4.4 mg, $\text{MnSO}_4 \cdot 4 \text{H}_2\text{O}$ 5.0 mg, CaCl_2 55.5 mg, yeast extract dehydrated, Difco, 50 mg and distilled water 1000 ml. pH about 5.8.

The cultures were grown in 300 ml Erlenmeyer flasks of Pyrex glass, each containing 20 ml, giving only a shallow layer to provide good aeration.

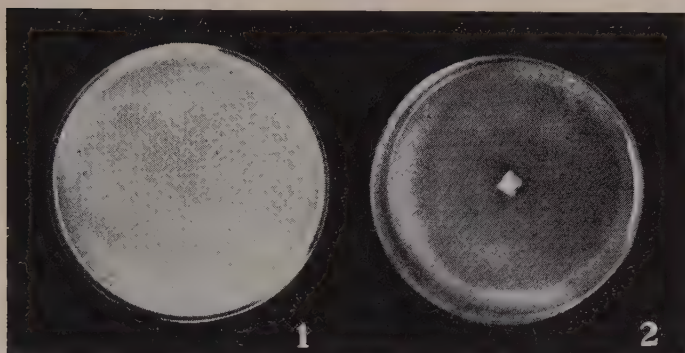


Fig. 1. Plates of cellulose agar. 1. Uninoculated; 2. Inoculated with *Tricholoma fumosum*, growing with scanty aerial hyphae, bringing about a transparent zone in the opaque agar.

Inoculation. Inoculations of flask cultures were made from mycelia, growing on »HAGEM-malt-agar« (cf. Fries 1941, p. 274) plates, by transferring two square pieces (2×2 mm) of the mycelium to each flask. The pieces were put floating on the surface of the culture medium, on opposite sides of a given flask. In this way it was insured that all parts of the cellulose medium would be invaded by the fungus as rapidly as possible.

In both *Tricholoma* species mycelial growth in different agar blocks usually does not start at the same rate. In order to obtain the most uniform growth rate possible, agar squares were placed on the surface of fresh »HAGEM-malt-agar« plates one day before the inoculation of the flasks. The mycelia which had attained approximately the same size were used as inocula.

The cultures were incubated at $+25^{\circ}$ C.

Determination of mycelial production. The mycelial yield could not be measured with any accuracy by weighing due to enmeshing of cellulose fibers with the hyphae. Therefore the total nitrogen of protein produced was determined by means of the titrimetric micro-Kjeldahl method. The procedure was performed in the manner recommended by Hiller, Plazin and van Slyke (1948) using mercury as a catalyst in the digestion.

Before the nitrogen determinations, the cellulose-mycelial mats were washed on glass filter crucibles (Jena 1 G 3) until the nitrogen, derived from the nutrient solution, was removed.

The time of incubation varied with the species studied and all species were incubated for time-periods which would give the maximum enzymic effect.

Tricholoma fumosum was cultivated for 25 or 30 days, the longer period

of incubation giving an average total N-value of 1.2 mg, corresponding to about 18 mg dry weight of mycelium of this species. (The nitrogen content of the mycelia grown under different conditions will be dealt with in a further paper.) pH of the solutions not below 4.0.

Tricholoma nudum was cultivated for 25 days with an average N-value of 0.46 mg, and for 35 days with 0.6 mg N, corresponding to about 7 respectively 9 mg dry weight of mycelium. pH not below 4.2. *Polyporus annosus* was cultivated for 12 days, giving an average N-value of 0.27 mg. pH 5.3.

Coniophora puteana was cultivated for 12 days, giving an average N-value of 0.28 mg. pH 5.0.

Enzyme preparations. The flask contents, including nutrient solution and mycelia, were poured into sterilized centrifuge tubes, centrifuged (3500 r.p.m., 8 cm radius to liquid surface) for 30–60 minutes until a sedimentation, sufficient to permit decanting of the cell-free solution.

Enzyme substrates. The cellulose was precipitated from a 2 per cent solution of swedish filter paper (Munktell n:r 3) in cuprammonium hydroxide by means of hydrochloric acid, mainly according to Mc Beth (1916). The precipitation was performed in nitrogen atmosphere thus avoiding the degradative effect of oxygen. The precipitated cellulose was liberated from Cu by washing with a 5 per cent Seignette salt solution (Staudinger 1936).

In some experiments an aqueous colloidal solution of cellulose micelles or micelle-aggregates, prepared from native cellulose, was used as substrate.

The author is greatly indebted to Dr. B. Rånby of the Institute of Physical Chemistry, Uppsala, for supplying the micelle solutions as well as for the D.P. determinations and x-ray diagrams. The micellar solution was prepared from wood cellulose (sulphitepulp of spruce wood, with 95.5 per cent α -cellulose from Bille-rud AB., Säfte) by boiling in a 2.5 N sulphuric acid for 8 hours. Concerning the details of the method by preparation and the properties of this sol, see Rånby 1949. The D.P. values were determined viscometrically, by the Ostwald viscometer method on nitrated samples (cf. Gralén and Rånby 1944) in acetone solution, $K_m = 9.6 \cdot 10^{-3}$.

Determination of the cellulolytic enzyme activity. A decrease of the extinction of the two different cellulose preparations caused by the influence of unheated cell-free solutions, has been taken as a measure of cellulolytic enzyme activity. The extinction has been determined in white light by means of the photometer, used by Åberg and Rodhe (1942). The extinction values have been expressed according to the formula $Z = (e_o - e_v) \cdot 10^3$ where $(e_o - e_v)$ is the difference between the initial extinction value of the culture tube with the cellulosic solution and that measured during the experiment. The various e-values have been calculated from the formula $\log \frac{I_o}{I}$. The tubes used were made of Jena glass with an inner diameter of 16–16.5 mm. Each tube was

first provided with 1 ml phosphate buffer of a concentration which would produce a 1/30 mol buffer of pH 5.0 on increasing the volume to the final 8 ml. The tubes were plugged and sterilized by autoclaving. After that, 5 ml of the enzyme preparation were transferred into the tubes and those, intended for controls, were placed in boiling water for 20 minutes. Finally each tube received 2 ml of the precipitated cellulose (n:r II was used in this investigation) giving an initial $e_0 \cdot 10^3$ value of 500—600. Toluol could not be used as an antiseptic because of its strongly interfering optical effect. However, any infection would immediately manifest itself as an increased extinction value. All tubes, running at least in duplicate, were shaken in a water bath of 30° C, and measured at intervals of several hours. Mean values are stated.

In certain cases the cellulolytic activity of the solutions was determined by their ability to form reducing breakdown products, the amounts of reducing sugars being determined according to Philipson (1943) or later according to Willstätter-Schudel (Bamann and Myrbäck 1941, s. 1077).

Experiments and results

Extinction value, dry matter concentration, and average degree of polymerization (D.P.). I and II are solutions of precipitated cellulose, prepared at two different times, and having an average D.P. of 266 and 178 respectively. III is primarily the same as II, but shaken for 90 hours with an enzyme mixture from *Tricholoma nudum*, resulting in a drop of D.P. to about 80. By stepped dilution of the solutions I, II, and III, three series of different concentrations were obtained. The curves obtained by photometric measurements of the three series are given in fig. 2.

It is obvious that the correlation between different concentrations, of one and the same cellulose solution and the corresponding Z values is represented by a nearly straight line. On the other hand, the same diagram shows that any absolute correlation between dry matter and extinction values does not exist, since the average size of cellulose particles causing the extinction, most likely varies with the degree of polymerization.

The effect of enzyme preparations on precipitated cellulose. A culture medium in which *Tricholoma fumosum* was grown for 25 days, was separated from the cellulose-mycelial mats, and transferred into test tubes with precipitated cellulose, 5 ml per tube, all in the above-mentioned manner. This medium caused a decrease of the extinction values of the solution. In the beginning, this decrease was rapid, as illustrated by fig. 3, curve C, showing a Z value of 195 after the first 12 hours. — The controls, with

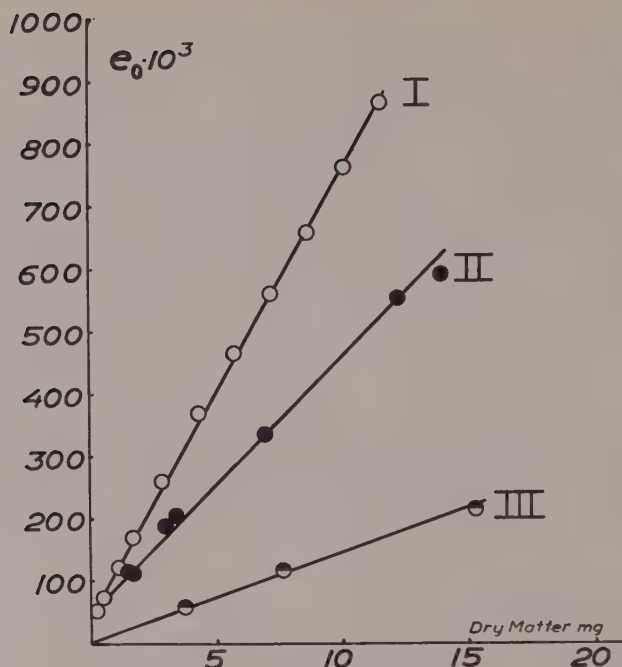


Fig. 2. Extinction values ($e_o \cdot 10^3$) of three solutions of precipitated cellulose from different average degree of polymerization (D.P.): I 266, II 178, and III about 80.

heated culture medium, did not show any extinction differences. — The two wood-rotting fungi, *Polyporus annosus* and *Coniophora puteana*, behaved in the same way. Their effect, however, particularly that of *Coniophora puteana* was more marked. The effect per mg nitrogen of mycelium seems to be greater of the wood-rotting fungi. The preparations, obtained from 12-days-old cultures, with an amount of protein-N produced per flask, equal to only about a quarter of that of *Tricholoma fumosum*, gav Z values of 195 and 280 respectively, after a treatment of only 8 hrs. as shown by fig. 4.

The results may be considered as a proof that an extracellular, cellulolytic enzyme is produced by the growing mycelia of these fungi. Certainly the bulk of cellulolytic enzymes, produced by the mycelium, is to be found in the liquid medium. Alternate freezing and thawing before centrifuging did not increase the cellulolytic activity of the culture medium.

The effect of enzyme preparations on micelle solution, some native celluloses, and lichenin. Curve M on fig. 3 illustrates the decrease in extinction value of the micelle solution, caused by the same *Tricholoma fumosum*-

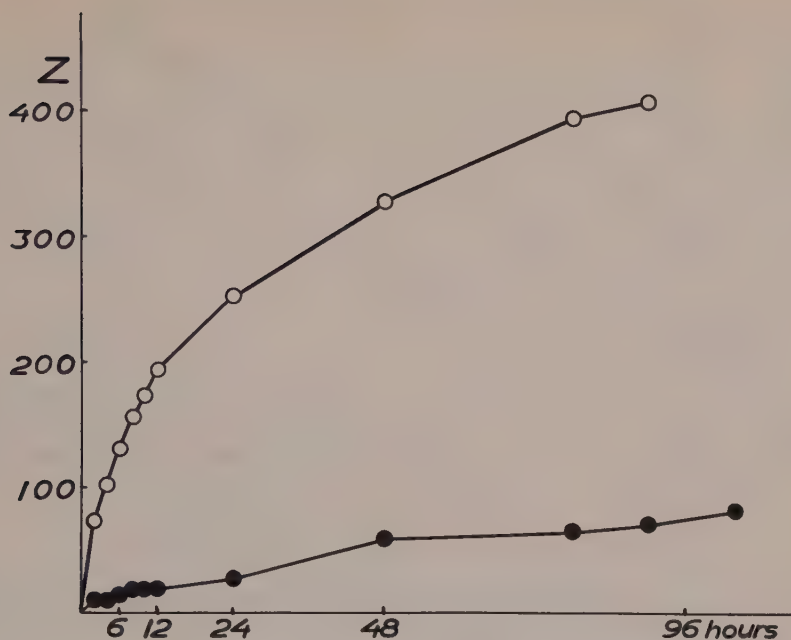


Fig. 3. Influence of cell-free nutrient solution from *Tricholoma fumosum* on the extinction values (Z) of a precipitated cellulose ○-○-○ (C) and a micellar solution ●-●-● (M).

preparation as that which affected the precipitated cellulose (see curve C). As it appears, the effect on the micelle solution was much less pronounced. The relative values of decrease, however, do not differ so greatly, since the initial average extinction value of the micelle tubes was only about a half of that of the precipitated cellulose solution. After 78 hours the relative values for the micelle solution and for the precipitated cellulose were found to be 19 and 61 per cent respectively. Enzyme preparations from *Tricholoma nudum* gave agreeing values, namely, 22 and 66 per cent after 72 hours. These results may be interpreted as showing that the chain-shortening and the splitting-off of sugar units from the micelles is either less intensive or optically less observable. In order to make it clear, the author followed the sugar production from micelles and from precipitated cellulose in the course of several hours. The former interpretation proved to be the more plausible (cf. fig. 5).

The effect of cell-free solutions (from *Tricholoma nudum*) on native cellulose was also studied in one experiment. Samples of 97.2 mg of wood-cellulose (the same as that used with micelle preparations), of de-waxed cotton, and of de-waxed and de-pectinized cotton were shaken with 30 ml

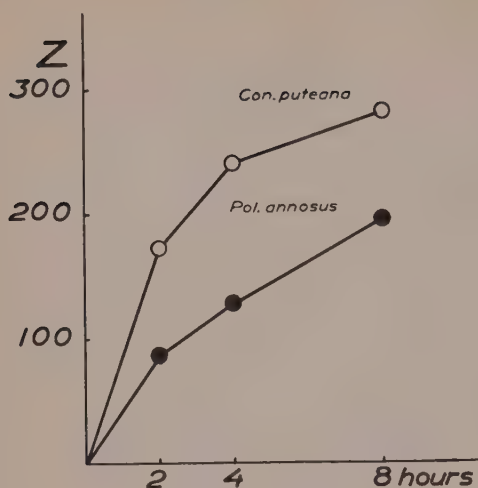


Fig. 4. Influence of cell-free nutrient solution from *Polyporus annosus* and *Coniophora puteana* on the extinction values (Z) of a precipitated cellulose.

enzyme preparation, unheated and heated, in large Jena-tubes (4×30 cm), with phosphate buffer and toluol as antiseptic, for 20 days. Then the reducing sugar was determined and calculated as glucose. The values, obtained after correction for reducing substances in the enzyme preparation, amounted to 6.5, 2.5, and 2.5 mg respectively with the unheated enzyme. The results indicate a slight decomposition of the native cellulose.

Lichenin samples of 32 mg per tube were shaken with 5 ml of the same enzyme preparation. After 8 hours, an average sugar yield of 17 mg was found.

Extinction values, decrease in cellulose, and increase in reducing sugar. A comparison between precipitated cellulose and micelle solution. As seen from fig. 1, a decrease in extinction values of the precipitated cellulose may be explained as a result of chain-breaking in central parts of the cellulose chains, without an attendant splitting-off of soluble products from the cellulose. In order to investigate if reducing sugars are formed during the enzyme treatment, the following experiment was performed. Test-tubes were provided with cellulosic material, phosphate buffer, and enzyme preparation in the usual manner and immediately measured photometrically. The contents of each of four test tubes were subsequently transferred to a centrifuge tube and centrifuged, after which the liquids were poured into flasks for sugar determination. The four test tubes were carefully rinsed with distilled water, the rinsing from each tube being poured into the corresponding centrifuge tube. The settled cellulose was agitated in the distilled water to remove the soluble products, and centrifuged again. The liquid in each tube was then added to its respective flask and the sugar

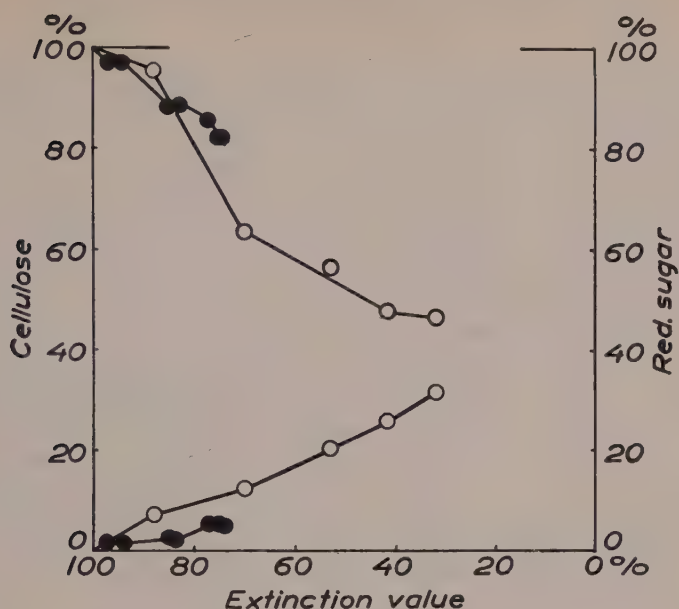


Fig. 5. Influence of cellulolytic enzyme mixtures on the percental decrease in precipitated cellulose $\circ-\circ-\circ$ or micellar solution $-●-●$ and increase of sugar formed, calculated as a percentage of the initial cellulosic material, plotted against the decreasing extinction values (Z), expressed in terms of per cent.

determined. The blank value, obtained in this manner, was subtracted from the following values. The residual cellulose was transferred quantitatively to weighing flasks, dried first at 80°C , later at 102°C , cooled above P_2O_5 , and weighed. The dry weight of the precipitated cellulose was 6.9 mg, that of the micelle solution 7.3 mg. The remaining tubes containing the precipitated cellulose and the micelle solution were measured photometrically, generally in quadruplicate, the precipitated cellulose after 4, 24, 48, 72, and 100 hours, the micelle solutions after 12, 36, 58, 92, 144, 192, and 264 hours. Simultaneously, the residual cellulose and the amounts of sugar formed were determined for every tube in the same manner. The results are illustrated by fig. 5, where the unfilled circles represent values of the precipitated cellulose, the filled circles those of the micelle solutions. The reducing sugar has been calculated as glucose anhydride ($\text{C}_6\text{H}_{10}\text{O}_5$), expressed in terms of per cent of the initial amount of cellulose.

Although an enzyme preparation from *Tricholoma nudum* was used in the case of precipitated cellulose, and one from *Tricholoma fumosum* was employed in the case of micellar solutions, a direct comparison is possible, these preparations being similar in their cellulolytic activity. Firstly, it is

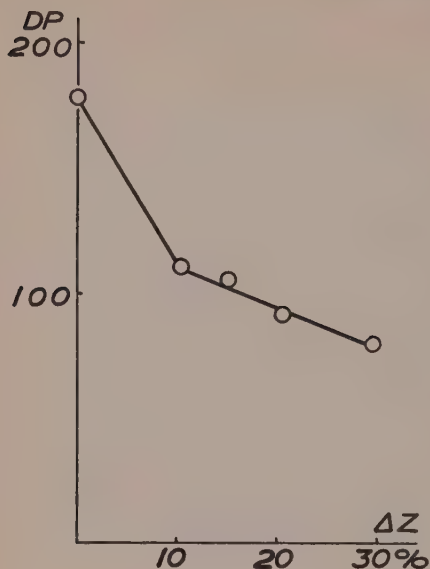


Fig. 6. Influence of an enzyme preparation from *Tricholoma nudum* on the drop in average degree of polymerization, D.P., of a solution of precipitated cellulose plotted against the decrease in extinction values, calculated as a percentage of the initial extinction value (ΔZ per cent).

obvious that soluble break-down products were actually formed in both cases (from micelles though in small quantities). The curve representing the decrease in cellulose, falls quite rapidly, while that representing sugar-formation rises more slowly. By rights, the reducing sugar should be calculated as cellobiose, since cellobiase seems to be lacking in the extra-cellular enzymes of the *Tricholoma* species. Between 4 and 24 hours, the precipitated-cellulose curve descends rapidly. This probably indicates that even higher saccarides as cellotriose and cellotetrose are split off. The products, however, have not yet been analysed. Any increase in reducing power of the residual precipitated cellulose (after a treatment of 24 hours) could not be established (by the method of Willstätter and Schudel).

Change of the average degree of polymerization (D.P.) under the influence of enzyme preparations. Samples of precipitated cellulose, 56.4 mg dry weight per Jena-tube, were shaken with 50 ml enzyme solution for periods of 8, 15, 36, and 45 hours. At these points, the average degree of polymerization was determined, and simultaneously photometric values were determined in test tubes, provided with equivalent amounts. Fig. 6 shows a rapid drop in the average D.P. during the first period, namely from 178—110. After that, the decrease proceeds gradually. The values of sugar formed, obtained after correction for reducing substances in the control, amounted to 2.6, 3.9, and 6.5 mg. The micelle solutions, on the other hand, present only very slight changes during the whole time of the experiment, decreasing from a D.P. of 68 to 65 and 64, after 65 and 260 hours treatment, respectively.

Discussion

The constantly lower reactivity of the micelle solution, as compared with that of precipitated cellulose, expressed in terms of extinction, sugar-formation, and changes of D.P. was expected as a consequence of earlier investigations. Thus, it has been suggested that an increased degree of crystallinity will give rise to a greater resistance to enzyme hydrolysis (e.g. Karrer et al. 1925) as has been proved in the case of acid hydrolysis.

The acid hydrolysis not only of native cellulose but also of regenerated cellulose shows a depolymerization, initially rapid, until micelle accumulation is reached. (Cf. Heuser 1944, Rånby and Ribí, 1950).

In the micelle preparation used, the crystalline material amounted to more than 70 per cent — since the original material contained 70 per cent of crystalline substance and the preparation involves a concentration of it — the rest consisting of the non-crystalline part of the micelle aggregates of the sol (cf. Ribí and Rånby, 1950). The changes of the micelle solutions noted above are probably due only to the degradation of the non-crystalline part. Thus, the state of crystallinity probably also affects the shape of the curve on fig. 6, showing the enzymic influence on precipitated cellulose. The first steep fall may be interpreted as depending on the presence of easily-attacked amorphous cellulose, whereas the following more gradual slope is probably due to the relative increase in the crystalline fraction. A certain inactivation of the enzyme might also, admittedly, influence the shape of the curve.

However, an x-ray diagram of precipitated cellulose, treated with an enzyme preparation for 8 hours already, not only showed more distinct lines but also additional lines, which had hitherto been invisible, thus indicating an increase in crystallinity.

The great velocity of the initial depolymerization and the decrease in extinction values without a corresponding increase in sugar formation, seems to indicate that the enzymes also cause cleavages in the *central* parts of the cellulose chains in the amorphous regions.

Summary

1. Cell-free solutions with cellulolytic activity were obtained from two soil-inhabiting hymenomycetes: *Tricholoma fumosum* and *Tricholoma nudum*.

2. The cellulolytic enzyme activity was measured photometrically.

3. In addition to the extinction measurements, sugar-formation and loss in cellulosic matter were determined.

4. Comparisons between the cellulolytic effect on precipitated cellulose and micelle solution were made. A correlation between increased degree of crystallinity and increased resistance against enzymic attack has been established.

5. The enzymes split off soluble reducing sugars as well as longer chains of glucose anhydride.

The author is indebted to Fil. mag. Mrs Ulla Björkman for valuable assistance.

References

- Åberg, B. & Rodhe, W.: Über die Milieufaktoren in einigen südschwedischen Seen. — *Symb. Bot. Ups.* 5, Nr. 3, 1—256. 1942.
- Bamann, E. & Myrbäck, K.: Die Methoden der Fermentforschung. — Leipzig 1941.
- Bose, S. R.: Enzymes of wood-rotting fungi. — *Erg. Enzymforsch.* 8: 267—276. 1939.
- Bose, S. R. & Sarkar, S. N.: Enzymes of some wood-rotting Polypores. — *Proc. Roy. Soc., Ser. B.*, 123: 193—213. 1937.
- Freeman, G. G., Baillie, A. J., & Macinnes, C. A.: Bacterial degradation of Sodium carboxymethylcellulose and methyl ethyl cellulose. — *Chemistry and Industry*, May: 279—282. 1948.
- Freudenberg, K. & Ploetz, Th.: Über die Verschiedenheit von Cellulase und Lichenase. 1. Mitteilung über den enzymatischen Abbau polymerer Kohlenhydrate. — *Z. physiol. Chemie* 259: 19—27. 1939.
- Fries, N.: Über die Sporenkeimung bei einigen Gasteromyceten und mykorrhizabildenden Hymenomyceten. — *Arch. f. Mikrobiol.* 12: 266—284. 1941.
- Gralén, N. & Rånby, B. G.: High-molecular wood cellulose. — »The Svedberg 1884 ³⁰/₁₀₀ 1944». 274—287. Uppsala 1944.
- Grassmann, W. & Rubenbauer, H.: Über Zellulase und Hemizellulase mit besonderer Berücksichtigung ihrer therapeutischen Anwendung. — *Münch. med. Wochenschr.* 78: 1817—1819. 1931.
- Grassmann, W., Stadler, R., & Bender, R.: Zur Specificität cellulose- und hemicellulose-spaltender Enzyme. 1. Mitt. über enzymatische Spaltung von Polysacchariden. — *Liebigs Ann. Chemie*, 502: 20—40. 1933 a.
- Grassmann, W., Zechmeister, L., Tóth, G., & Stadler, R.: Über den enzymatischen Abbau der Cellulose und ihrer Spaltprodukte. 2. Mitt. über enzymatische Spaltung von Polysacchariden. — *Ib.* 503: 167—179. 1933 b.
- Heuser, E.: The chemistry of cellulose. — New York 1944.
- Hiller, A., Plazin, J., & van Slyke, D. D.: A study of conditions for Kjeldahl determination of nitrogen in proteins. — *J. Biol. Chem.*, 176: 1401—1420. 1948.
- Karrer, P., Schubert, P., & Wehrli, W.: Polysaccharide XXXIII. Über enzymatischen Abbau von Kunstseide und nativer Cellulose. — *Helv. Chim. Acta* 8: 797—810. 1925.
- Kellerman, K. F. & Mc Beth, I. G.: The fermentation of cellulose. — *Zentralbl. Bakt.* II Abt. 34: 485—494. 1912.
- Levinson, H. S. & Reese, E. T.: Enzymatic hydrolysis of soluble cellulose derivatives as measured by changes in viscosity. — *Am. J. Bot.* 36: 811 (Abstract.). 1949.
- Mc Beth, I. G.: Studies on the decomposition of cellulose in soils. — *Soil Science* 1: 437—487. 1916.

- Meyer, K. H. & Gürtler, P.: Recherches sur l'amidon XXXI. La constitution de la lichénine. — *Helv. Chim. Acta* 30: 751—761. 1947.
- Norkrans, B.: Om mykorrhizabildande och rent saprofytiska *Tricholoma*-arters fysiologi. — *Svensk Bot. Tidskr.* 38: 121—122. 1944.
- Some mycorrhizaforming *Tricholoma* species. — *Svensk Bot. Tidskr.* 43: 485—490. 1949.
- Philipson, T.: Über die quantitative Bestimmung reduzierender Zucker. — *Arkiv f. kemi*, 16 A, Nr. 22, p. 19. Uppsala 1943.
- Ploetz, Th.: Über einige Enzyme des Hausschwammes (*Merulius lacrimans*) 2. Mitt. über den enzymatischen Abbau polymerer Kohlenhydrate. — *Z. physiol. Chemie* 261: 183—188. 1939.
- Rånby, B. G.: Aqueous colloidal solutions of cellulose micelles. — *Acta Chem. Scand.* 3: 649—650. 1949.
- Rånby, B. G. & Ribí, E.: Über den Feinbau der Zellulose. — *Experientia* VI: 1, 12. 1950.
- Reese, Elwyn T.: Aerobic decomposition of cellulose by micro-organisms at temperatures above 40° C. — *Am. J. Bot.* 33, 831 (Abstract). 1946.
- Ribí, E. & Rånby, B. G.: Zur elektronenmikroskopischen Präparation von Kolloiden. — *Experientia* VI: 1, 27. 1950.
- Saunders, P. R., Sin, R. G. H., & Genest, R. N.: A cellulolytic enzyme preparation from *Myrothecium Verrucaria*. — *J. Biol. Chem.* 174: 697—703. 1948.
- Staudinger, H. & Feuerstein, K.: Über hochpolymeren Verbindungen. 147 Mitt. Über den Polymerisationsgrad natürlicher und technischer Cellulosen. — *Liebigs Ann. Chemie* 526: 72—102. 1936.

Transfer of Radioactive Phosphorus to Pine Seedlings by Means of Mycorrhizal Hyphae

By

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(Received March 16, 1950)

It has been concluded by several investigators during the last decades that tree mycorrhizae are nutrient absorbing structures (Melin, 6, 7, 8, Hatch, 3, 4, Björkman, 1, 2, and others). However, no direct measurements have been made concerning absorption and transfer of elements by means of mycorrhizal hyphae.

Recently Kramer and Wilbur (5) studied absorption of radioactive phosphorus by mycorrhizal and nonmycorrhizal portions of pine roots. Pieces of roots obtained from potted pine seedlings were exposed for 3 or 4 hours to solutions containing P^{32} in amounts varying from 100—500 $\mu\text{c}/\text{l}$ in various experiments. According to the subsequent measurements of the radioactivity of the roots, the mycorrhizal portions seemed to accumulate larger quantities of phosphorus than nonmycorrhizal portions. However, it was not demonstrated that the phosphorus of mycorrhizae had been absorbed by the mycorrhizal hyphae.

For our experiments reported below, the leading principle was to make arrangements, if possible, for exposing only the mycorrhizal hyphae to the isotope. In preliminary tests, the transport of P^{32} through mycorrhizal hyphae was studied in the following way. Small glass cups were placed excentrically in petri dishes containing agar substrate. These cups were then given, aseptically, a suitable nutrient solution. Mycelia from pure cultures of various Hymenomycetes, forming mycorrhizae with pine — *Amanita muscaria* (L.) Fr., *Boletus luteus* (L.) Fr., *B. variegatus* (Sw.) Fr., *Lactarius deliciosus* (L.) Fr. or *Tricholoma imbricatum* (Fr.) — were introduced in the dishes. After some time, hyphae had grown over the edges of the cups and

down to the nutrient solution. An acid solution of P^{32} was put in the cups and the activity of the mycelium on the agar was measured at desired intervals with a Geiger-Müller counter. From these experiments the following results were obtained. 1) It was possible for fungus hyphae to grow over a barrier of glass of considerable height (up to 15 mm). 2) The hyphae transported P^{32} in measurable quantities. 3) The quantities transported were proportional to the distance from the P^{32} -source but did not appear to be influenced by amounts above $0.1 \mu\text{c}$ in the source.

In our main experiments, we used a modification of the pure culture method introduced by Melin (7, 9). On the bottom of 500-ml wide-necked Erlenmeyer flasks (Pyrex glass), near the wall, were placed cylindrical glass cups with an inner diameter of 3.5 cm and a height of 1.8 cm (Fig. 1 B). Then 160 g purified sand (Melin, 9, p. 1082) was put in the outer vessel and 40 g in the cup. These quantities allowed the cup edges to be 3—7 mm above the sand level. Nutrient solution was added to both vessels in amounts 0.25 ml corresponding to 1 g sand. The basal medium contained, per litre distilled water:

K_2HPO_4	0.5	g
CaCl_2	0.05	g
NaCl	0.025	g
$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	0.15	g
$(\text{NH}_4)_2\text{HPO}_4$	0.25	g
Fe-citrate (1 % solt.)	1.2	ml
Glucose	2.5	g

The nutrient solution was supplemented with thiamine (15 γ per litre).

At the end of April one aseptic seedling of *Pinus silvestris* L. was introduced into each of the Erlenmeyer flasks and after 4 months the cups were inoculated with mycelium of *Boletus variegatus*. A couple of weeks later the hyphae had grown over the cup edges into the sand of the Erlenmeyer flasks and after two months mycorrhizae were very well developed (Fig. 1). The cultures were then ready for the treatment with P^{32} . Only flasks which had been proved to be free from air infections were used. The isotope, essentially carrierfree, was obtained in Na_2HPO_4 -solution. It was added to the cups in desired activities varying from $1 \mu\text{c}$ to 1 mc in various experiments. After varied periods of exposure, ranging from a few hours to several days, the experiments were stopped and the activities of the seedlings were measured. Before taking out the seedling from the flask, the fungus mycelium was cut off at the edge of the cup and the cup was cautiously removed by means of a special arrangement. The roots were carefully washed and freed from sand and desired segments of roots, stem and needles were cut off and their

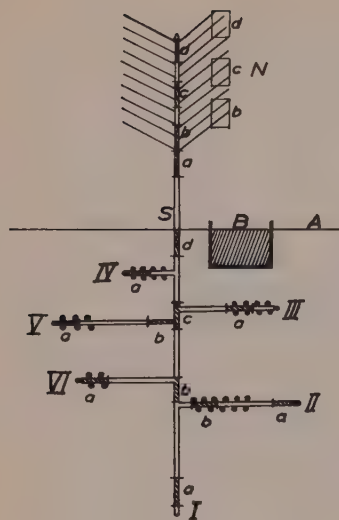


Fig. 1. Schematic drawing of pine seedling used for radioactive measurements given in Table 1. A=Sand level; B=Glass cup; S=Stem; N=Needles; I=Main root; II—VI=Lateral roots (black dots indicate well-developed mycorrhizae); a—d=Segments measured for radioactivity. (Cf. tables 1—2.)

volumes measured with a modified volumenometer. The segments were ground in mortars, after which their activity was measured with a Geiger-Müller counter.

As is understood, the isotope may also diffuse from the hyphae into the sand of the Erlenmeyer flasks and then be absorbed directly by the roots. Therefore it was necessary to know also the radioactivity of the sand in the flasks. This was calculated from measurements of the radioactivity of water extract (cf. note of table 1).

The experimental results are illustrated by Table 1, in which are given values of measured radioactivities and volumes of one seedling schematically drawn in fig. 1. The mycelium of the cup had been exposed for two days to 0.1 mc P^{32} . The radioactivity values are given with correction for absorption in the ground sample and for the decay. In Table 2 all of the figures are calculated for 0.1 ml as unit volume to make the results comparable.

The results may be summarized as follows. The measurements of radioactivity in the pine seedlings showed that the non-mycorrhizal tips of the main and of the lateral roots contained only very small amounts of P^{32} , whereas segments bearing mycorrhizae contained considerable quantities of the isotope. Since the mycelia in the cups, when exposed to the radioactive solution, still had intact hyphal connections with the mycorrhizae, it seems clear that the radioactive component had been transported from its source to the mycorrhizae by the *Boletus* hyphae. The relatively high activities of older non-mycorrhizal portions of lateral roots bearing mycorrhizae in their

Table 1. *Measured activities and volumes of segments indicated in Fig. 1.*

Index given in Fig. 1	Imp./min.				Volume ml			
	a	b	c	d	a	b	c	d
I	15.3	38.5	64.9	89.2	0.06	0.065	0.07	0.07
II	25.7	50.6			0.04	0.03		
III	80.6				0.04			
IV	81.8				0.03			
V	65.6	53.2			0.04	0.06		
VI	57.4				0.04			
S	68.5	65.4	53.8	52.3	0.09	0.09	0.08	0.09
N		68.1	61.0	55.7		0.19	0.17	0.19

The activity of the sand was 15.0 imp./min./0.1 ml.

younger parts (cf. Table 1, V b) demonstrated that the isotope had also been transferred to the cells of the higher symbiont from the fungal partner. The isotope had then been translocated to the main roots, stems and needles. Already after treatment for 7.5 hours with radioactive phosphorus the isotope could be traced in the pine needles.

From the experiments referred to above, it has been demonstrated that Boletus variegatus (Sw.) Fr., as fungal symbiont, is able to transfer phosphorus to the pine roots.

The measurements of radioactivity were made at the Institute of Physics of the University. We wish to express our appreciation to Prof. Axel Lindh for generous cooperation and to Dr. Gösta Carlsson for guidance and assistance.

Radioactive phosphorus was obtained from the Nobel Institute for Physics, Academy of Science, Stockholm. We are indebted to Prof. Manne Siegbahn for his valuable help.

Table 2. *Activities and corresponding amounts of P^{32} calculated for 0.1 ml as unit volume.*

Index given in Fig. 1	Imp./min./0.1 ml				$P^{32}/0.1 \text{ ml} \times 10^4 \text{ m}\gamma$			
	a	b	c	d	a	b	c	d
I	25.5	59.2	92.7	127.4	14.5	33.7	52.6	72.1
II	64.3	168.7			36.5	95.7		
III	201.5				114.3			
IV	272.7				154.7			
V	164.0	88.7			93.0	50.3		
VI	143.5				81.5			
S	76.1	72.7	67.3	58.1	43.2	41.2	38.3	33.0
N		35.8	35.8	29.3		20.4	20.4	16.6

References

1. Björkman, E.: Über die Bedingungen der Mykorrhizabildung bei Kiefer und Fichte. — Symb. Bot. Ups. VI: 2. 1942.
2. — The ecological significance of the ectotrophic mycorrhizal association in forest trees. — Svensk Bot. Tidskr., 43. 1949.
3. Hatch, A. B.: The role of mycorrhizae in afforestation. — Journ. of Forest., 34. 1936.
4. — The physical basis of mycotrophy in Pinus. — The Black Rock Forest Bull., 6. 1937.
5. Kramer, P. J. & Wilbur, K. M.: Absorption of radioactive phosphorus by mycorrhizal roots of pine. — Science, 110. 1949.
6. Melin, E.: Studier över de norrländska myrmarkernas vegetation med särskild hänsyn till deras skogsvegetation efter torrläggning. — Norrl. Handbibl. 7. Uppsala 1917.
7. — Experimentelle Untersuchungen über die Konstitution und Ökologie der Mykorrhizen von Pinus silvestris L. und Picea Abies (L.) Karst. — Mykol. Unters. u. Ber. von R. Falck, 2. 1923.
8. — Untersuchungen über die Bedeutung der Baummykorrhiza. — Jena 1925.
9. — Methoden der experimentellen Untersuchung mykotropher Pflanzen. — Abderhaldens Handbuch der biologischen Arbeitsmethoden, Abt. XI, 4. 1936.

The Nitrogen Nutrition and Vitamin Requirement of *Ophiostoma pini*

By

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The common blueing fungus *Ophiostoma pini* (Münch) Nannf. is considered to belong to the auxo-heterotrophic organisms, i.e., organisms which are not able to grow in nutrient solutions containing pure inorganic salts and simple carbohydrates but require, in addition to these, small amounts of certain organic compounds, so called growth factors (Schopfer 1943). *O. pini* is auxo-heterotrophic with respect to the vitamins aneurine (B_1) and biotin (Fries 1943). The conception of auxo-heterotrophy is used in a rather wide sense. Both organisms which have lost their capacity to synthesize growth factors genetically and organisms which have not lost it genetically but in which it is inhibited in its action are regarded as auxo-heterotrophic. If the inhibition is not complete, a small quantity of growth substances may be formed. If this inhibition is complete, it leads practically to the same result as in the case where the gene is entirely absent. This inhibition may be dependent on the medium, in which case a modification of the medium permits the organism to synthesize its necessary growth substances.

Since *Ophiostoma pini*, after the addition of aneurine and biotin, is able to produce sufficient amounts of dry matter in the common nutrient solutions (with NH_4Cl resp. NH_4 -tartrate and glucose), but cannot produce normal pigment or perithecia, the following questions arise 1) is *O. pini* able to develop normally on these substrates after the addition of other vitamins and metals, and 2) are there other macro-nutrients, more suitable for the normal development of *O. pini* and are its vitamin requirements on these the same?

In some cases the nitrogen source is known to have a certain effect on

the vitamin requirements of the organisms qualitatively (Leonian and Lilly 1938, Fries 1943) as well as quantitatively (Schopfer 1943, p. 103). Even the metals may influence the vitamin requirements (Steinberg 1936, Leonian and Lilly 1938). In the following work the influence of the nitrogen and metal constituents to the growth of *O. pini* is studied.

Method and material

The strain of *O. pini* was isolated from infected pine wood and was during the experiments 0.5—1.5 years old. The stock cultures were growing well on 2.5 per cent malt extract agar, sporulated richly, produced numerous perithecia, and were normally pigmented.

For tests, 100 ml Erlenmeyer flasks (Pyrex) were used, each containing 25 ml of nutrient solution. Four parallel tests were conducted with each sample. Stock solution with the following composition was used:

Solution A:

1000 ml H₂O

10.0 g glucose puriss.

2.0 g KH₂PO₄

0.5 g MgSO₄

2.0 g NH₄Cl or responding amounts of some other nitrogen compound.

Microelements were added as follows:

H ₃ BO ₄	0.0025	ppm
CuSO ₄	0.01	»
FeCl ₃	0.01	»
MnSO ₄	0.005	»
ZnSO ₄	0.005	»
(NH ₄) ₆ Mo ₇ O ₂₄	..	0.005	»

Ca and Na were added as chlorides to a conc. of 0.1 per cent. The vitamins and microelements were added before sterilization in an autoclave. With some nutrient media, where no satisfying growth occurred, double tests were carried out, supplying vitamins after sterilization and sterilizing with Seitz filter. The pH in most media, after sterilization, was 4.0—4.5. In tests where the growth of the fungus with and without the microelements was compared, water redistilled in Pyrex-glass distilling apparatus was used; the other tests were conducted with once distilled water. As water, distilled in Pyrex-glass tubes is not entirely boron-free, the series entitled as »without microelements» in the following tables ought to be considered as containing boron as contaminant.

Nutrient solutions were inoculated with 1 ml spore suspensions containing 1000 spores per ml. This suspension was prepared by growing the fungus in test-tubes on 1 per cent malt extract agar. To mature cultures, approximately 5 ml sterile distilled water was added and the tubes swirled lightly. Thus a solution containing 5—10 mill. spores per ml was provided, which was diluted 5000—10000 times. After such a procedure the amount of substances possibly transferred from the agar cultures to the test flasks would be exceedingly small. Since the spores of

Table 1. *The dry matter production of O. pini after inoculation with different amounts of spores.*

Nutrient solution	Vitamin	Dry matter after 20 days, mg			
		1,000,000 spores	100,000 spores	10,000 spores	1,000 spores
Solution A with NH ₄ -tartrate + Na + Ca dist. water	—	—	—	—	—
	aneurine 1 γ	+	+	+	+
	aneurine 1 γ + biotin 0.1 γ	7.7	9.4	10.8	13.5
	malt extract 0.2 p.c.	22.4	19.5	19.3	23.3
	malt extract 0.2 p.c.	7.8	7.6	9.0	8.3

Table 2. *The growth of O. pini in malt extract solutions.*

Growth of <i>O. pini</i>	Malt extract per cent						
	0.01	0.05	0.1	0.5	1.0	2.5	5.0
Dry matter, mg	0.8	2.0	3.1	31.7	42.5	71.9	90.8
Colour	9	9	9	10	10	10	10
Conidia	+++	+++	+++	+++	+++	+++	+++
Perithecia	—	+	+	+++	+++	+++	+++

O. pini are able to grow out yeast-like and as the producing of spores begins already on the first mycelial fragments, after some weeks no differences in the development of cultures infected with unequal amounts of spores are found (Table 1).

The growth of *O. pini* in various media is estimated by its dry matter production. Spore and perithecia production is estimated in 3 degrees: poor (=1), moderate (=2), and rich (=3 t.ex. in tab. 2). In order to estimate the pigment production of the mycelium more objectively, the method employed by Rennerfelt (1945) for measuring the intensity of blueing was used. Thus, series of briquettes of wood flour were made containing a mixture of different proportions of charcoal and dyes and the colour of the mycelium was compared with them. A scale of 10 degrees was used, the briquettes containing dyes 0 (=degree 1); 0.001 (=degree 2 etc.); 0.0025; 0.005; 0.015; 0.025; 0.075; 0.15; 0.5; and 1.0 (=10) parts of weight of the wood flour.

The growth of *O. pini* and the nitrogen source

O. pini is able to develop normally in nutrient solutions containing pure malt extract (Table 2). Normal pigment and perithecia are produced even in solutions containing 0.05 per cent malt extract. On synthetic media, the development of *O. pini* is as follows:

Table 3. *Dry matter production in mg of O. pini on different nitrogen sources.*

N-source	Vitamins									
	without microelements					microelements added				
	—	aneurine 1 γ	biotin 0.1 γ	aneurine + biotin 1 γ + 0.1 γ	malt 2.5 mg	—	aneurine 1 γ	biotin 0.1 γ	aneurine + biotin 1 γ + 0.1 γ	malt 2.5 mg
KNO ₃	0.3	4.6	1.4	14.5	0.2	+	4.2	1.2		15.2
Ca(NO ₃) ₂	0.8	4.6	1.1	17.4	2.4	+	3.6	1.0		14.7
NaNO ₃	+	1.0	0.6	16.4	2.0	+	2.8	1.1		16.7
NH ₄ NO ₃	—	6.1	0.4	18.4	1.0	—	—	0.2		17.7
NH ₄ Cl	—	0.8	0.5	5.5	1.4	—	—	—	8.7	—
» 1	—	1.0	0.5	8.0	1.2	—	—	—		—
» 2	—	0.4	4.0	5.5	2.2	—	—	—		—
(NH ₄) ₂ SO ₄	—	+	+	12.4	+	—	+	+	18.8	15.8
» 1	—	+	+	10.4	+	—	+	+		14.2
» 2	—	0.4	+	8.8	0.8	—	+	—		10.5
(NH ₄) ₂ HPO ₄	—	+	+	13.7	+	—	+	+		+
NH ₄ -tartrate	—	2.8	3.0	16.8	0.7	—	3.7	2.5	22.4	0.6
asparagine	—	+	—	13.8	+	—	2.0	+	13.5	+
carbamide	—	1.8	1.2	10.2	+	—	0.8	0.2		+

¹ Vitamins added after sterilization.² Sterilized with Seitz filter.a. *Production of dry matter*

In table 3 the dry matter production of *O. pini* after 30 days is given. Without adding any vitamins, some growth occurs on the nitrate media. With microelements on vitamin-free media, the growth is no better, but in one case sterile perithecia were produced. Spores were readily germinated in all solutions used. After the addition of aneurine and biotin, *O. pini* is capable of producing dry matter in all media rather well with the exception of NH₄Cl. Best growth occurred with nitrates, asparagine, and NH₄-tartrate. In no case, however, was the dry matter production comparable with the growth in 0.5 per cent malt extract, where the total amount of nutrients was only $\frac{1}{3}$ of the mineral media. Aneurine and biotin were added in super-optimal concentrations, the ratio mycelium weight/vitamin being 20,000 for aneurine and 200,000 for biotin. After Fries (1948, p. 127) the economic coefficient for aneurine is 400,000—2,500,000 and for biotin 4,000,000—17,000,000. Increasing and decreasing the concentrations of the vitamins 10 times had no effect of the growth of *O. pini*. After the addition of only aneurine or biotin, the growth was, depending on the medium, better than without vitamins. With only aneurine, the dry matter production was better than with only biotin, reaching in KNO₃, Ca(NO₃)₂, and asparagine $\frac{1}{4}$ — $\frac{1}{3}$ of the dry matter production with both vitamins. Thus the aneurine and

Table 4. *The pigment production of O. pini in different nitrogen media. Scale 1—10.*

N-source	Vitamins									
	without microelements					microelements added				
	—	aneurine 1 γ	biotin 0.1 γ	aneurine + biotin 1 γ + 0.1 γ	malt 2.5 mg	—	aneurine 1 γ	biotin 0.1 γ	aneurine + biotin 1 γ + 0.1 γ	malt 2.5 mg
KNO ₃	(+)	1	3	9—10	—	—	1	1	—	7
Ca(NO ₃) ₂	—	2	5	10	5	—	—	1	—	10
NaNO ₃	—	1	3	9—10	9—10	—	1	1	—	10
NH ₄ NO ₃	—	—	5	5—8	—	—	—	—	—	5—6
NH ₄ Cl	—	—	3	2—3	—	—	—	—	1—2	—
» 1	—	—	3	2—3	—	—	—	—	—	—
» 2	—	—	3	2	—	—	—	—	—	—
(NH ₄) ₂ SO ₄	—	—	—	5—6	—	—	—	—	2	4
» 1	—	—	—	5	—	—	—	—	—	4
» 2	—	—	—	5	—	—	—	—	—	4
(NH ₄) ₂ HPO ₄	—	—	3	8	—	—	—	—	—	—
NH ₄ -tartrate	(+)	—	—	8	—	—	—	—	7—8	—
asparagine	—	—	3—5	8	—	—	—	—	—	—
carbamide	—	—	—	3	—	—	—	—	—	—

¹ Vitamins added after sterilization.² Sterilized with Seitz filter.

biotin deficiency in *O. pini* is not complete but is dependent on the nitrogen source.

After adding small amounts (0.01 per cent) of malt extract to mineral media, the dry matter production was still insufficient. After the addition to mineral media of 0.01 per cent malt extract and microelements, normal growth occurred with nitrates and (NH₄)₂SO₄ but not with other media. The addition of only microelements to mineral media or of microelements with only one of the vitamins had no effect on the dry matter production.

Thus in pure mineral media *O. pini* is not capable of synthesizing sufficient amounts of aneurine and biotin, and requires their addition. After adding 0.01 per cent malt, the vitamin supply is still insufficient but it seems that going out from the compounds the fungus receives in the malt extract, with the help of microelements, the fungus is capable to synthesize the required vitamins in sufficient amounts.

b. *The pigment production on different media*

Normally the substrate mycelium of *O. pini* is dark blackish-brown. The pigment production begins, depending on the substratum, 4—5 days after the germination of the spores. The advancing hyphae and air mycelium

Table 5. *Mycelial development, sporulation and perithecia production of O. pini in different nitrogen sources.*

N-source	Vitamins									
	without microelements					microelements added				
	—	aneurine 1 γ	biotin 0.1 γ	aneurine + biotin 1 γ + 0.1 γ	malt 2.5 mg	—	aneurine 1 γ	biotin 0.1 γ	aneurine + biotin 1 γ + 0.1 γ	malt 2.5 mg
Ca(NO ₃) ₂										
per cent normal cells	100	100	100	100	90	100	100	100	100	100
sporulating	+	+++	+	+++	++	++	++	+	+++	++
perithecia	—	—	—	p	—	p	—	—	p	p
(NH ₄) ₂ SO ₄										
p.c. normal cells	0	20	90	90	0	0	20	80	80	40
sporulating	—	++	+	+	+	—	++	+	++	+
perithecia	—	—	—	—	—	—	—	—	—	—
NH ₄ -tartrate										
p.c. normal cells	0	50	50	100	50	0	50	50	80	50
sporulating	+	+++	+	+++	++	+	+++	+	++	++
perithecia	—	—	—	—	—	—	—	—	—	—
asparagine										
p.c. normal cells	0	20	20	60	20	0	20	20	60	20
sporulating	+	++	++	++	+	+	++	+	++	+
perithecia	—	—	—	—	—	—	—	—	—	—

remains colourless. It is generally expressed that the pigment production begins after the mycelium has reached a certain stage of maturity. The pigment production does not, however, depend on the age or quantity of mycelium. In media with NH₄Cl and (NH₄)₂SO₄ no pigment is produced (Tab. 4), although the mycelium growth is sufficient. In media with nitrates and asparagine, pigmentation begins already with minimal growth of the mycelium. Normal blackish-brown pigment occurs only in media with KNO₃, Ca(NO₃)₂, and NaNO₃ after the addition of aneurine and biotin resp. malt and microelements. In the former media, no other metals than K and Mg or Ca, K and Mg or Na, K and Mg are present. In media with NH₃-salts, where no pigmentation occurs, even the presence of the former and other metals has no effect.

Several fungi have been found to require for their pigment production certain metal ions (Bortels 1927, Steinberg 1945 a.o.). Metz (1930), in a study on the pigment production of several fungi divided his material into two groups: 1) brightly coloured fungi such as the *Aspergillus* and *Penicillium* species, which often required the presence of some metal ion for the pigment formation; and 2) dark coloured mycelia, such as *Macrosporium* and *Phoma*, where no special metals were required. *O. pini* resembles the last group. As the pigment production occurs only with those substrata where the fungus is even otherwise growing normally, it seems that the pigment



Fig. 1. Normal hyphae of *O. pini*.

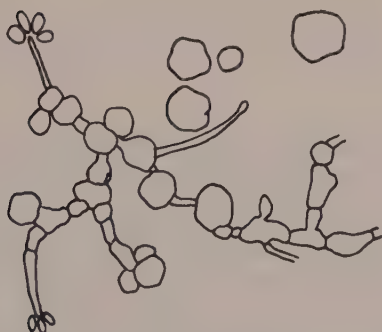


Fig. 2. Hyphae of *O. pini* in nutrient solution with asparagine.

production is closely bound to some assimilation process. Since the submerge mycelium is often of lighter colour and darkens upon being exposed to the air, it is possible that some oxidation process causes the pigment formation. The entirely colourless mycelia, however, do not darken in the air.

c. Mycelial development, sporulating, and perithecia formation

In other media than nitrates, the normal development of *O. pini* is disturbed. This is well illustrated by the appearance of the mycelium. In almost all cases, normal long and narrow cells are formed on nitrates (Table 5, fig. 1). In other substrates the cells are short, even round, and of much larger dimensions (fig. 2). In single cases, as on $(\text{NH}_4)_2\text{HPO}_4$ and NH_4 -tartrate, normal cells are formed after the addition of vitamins. The addition of only biotin often gives better results than the addition of only aneurine.

O. pini normally produces very rich conidia on the mycelium. In flask cultures, spore production begins already on hyphae consisting of only 5—6 cells even without the vitamins. The addition of aneurine markedly increases the spore formation, whereas the addition of biotin has no marked

Table 6. *Dry matter production in mg at varying vitamin and nutrient solution concentrations. N-source: $\text{Ca}(\text{NO}_3)_2$; glucose 2 per cent.*

Ca-nitrate concentration per cent	Vitamins						
	aneurine				biotin		aneurine + biotin
	1 γ	10 γ	100 γ	500 γ	0.1 γ	1.0 γ	1 γ + 0.1 γ
5	—	—	—	—	—	—	+
2	—	—	—	+	—	—	+
1	—	—	—	+	—	—	12.5
0.4	+	+	+	+	—	—	13.7
0.1	+	+	+	+	+	+	14.6
0.05	+	+	+	2.1	+	+	9.9
0.001	+	+	+	2.0	+	+	7.4
0.0005	+	+	+	+	+	+	+

effect. On substrates where the growth is otherwise normal, the spore formation is also very rich.

Perithecia are produced in pure malt extract solutions already at a minimal dry matter production (0.05 per cent malt extract). In synthetic media, perithecia were produced in KNO_3 , $\text{Ca}(\text{NO}_3)_2$ and NaNO_3 solutions after the addition of aneurine and biotin. In $\text{Ca}(\text{NO}_3)_2$ and NaNO_3 , sterile perithecia were produced even after the addition of 0.01 per cent malt and microelements without the vitamins. On other media, even after the addition of combinations of other vitamins (nicotinic acid, pantothenic acid, oleic acid, aspartic acid) and metals, no results were achieved.

Vitamin requirements and the concentration of solutions

One essential condition in the mycelium development is the nitrogen content of the medium. The nitrogen content of the medium may have a quantitative effect on the aneurine requirement (Schopfer 1945, p. 105). Moreover, organisms which have apparently lost their ability to synthesize a vitamin may regain their synthetic ability as a result of a quantitative variation of the medium (Robbins & Kavanagh 1938). The synthesizing power of *O. pini*, in the most favourable medium $\text{Ca}(\text{NO}_3)_2$ could not be reestablished after a quantitative variation of the nitrogen content (Table 6).

Replacement of biotin by oleic acid and aspartic acid

Biotin is supposed to take part in nitrogen assimilation. Winzler, Burk, and Du Vigneaud (1944) showed that after the addition of biotin a rapid increase in fermentation and growth took place, but only on NH_3 -media.

Table 7. Dry matter production in mg with oleic acid and aspartic acid.

N-source	Vitamins			
	oleic acid 10 γ	aspartic acid 10 γ	aneurine + oleic acid 1 γ + 10 γ	aneurine + aspar- tic acid 1 γ + 10 γ
Ca(NO ₃) ₂	1.7	0.9	14.3 perithecia norm. colour	14.4 perithecia norm. colour
NH ₄ Cl	—	—	17.1 colourless	17.7 colourless
NH ₄ -tartrate	+	+	13.2 colourless	12.8 colourless
asparagine.....	—	—	18.8 colourless	18.1 colourless

The authors suggested that biotin in some way brings about the NH₃-assimilation. In the case of *O. pini*, even on pure nitrate substrates biotin is indispensable for a normal growth. According to Potter and Elvehjem (1948), biotin brings about a synthesis of aspartic acid by catalyzing the carboxylation of pyruvate to form oxaloacetate which can be transaminated to aspartic acid. Other experiments indicate the same. Perlman (1948) showed that the addition of aspartic acid decreases the biotin requirement of *Memnoniella echinata* and *Stachybotrys atra*. In *Lactobacillus arabinosus* (Potter & Elvehjem l.c.), biotin could be completely replaced by aspartic or oleic acid.

In *O. pini*, biotin likewise was entirely replacable by aspartic acid and oleic acid (Table 7). On nitrate media a normal growth, pigmentation, and perithecia formation was observed after the addition of aneurine+aspartic acid or aneurine+oleic acid. After adding aspartic acid or oleic acid without aneurine, a minimal growth appeared only on nitrate media.

Summary

1. *O. pini* is partially heterotrophic with respect to aneurine and biotin. In favourable substrates, such as nitrates, especially Ca(NO₃)₂, it is able to produce some mycelium and sterile perithecia after the addition of Na, Zn, Cu, Fe, B, Mn, and Mo, without any vitamins.

2. By adding aneurine and biotin a normal growth, pigmentation, and perithecia production occurs on media composed of glucose, some nitrate, KH₂PO₄, and MgSO₄. No other metals or compounds were required.

3. Malt extract in small amounts (0.01 per cent) is not able to produce normal growth on any of the nitrogen media tested. After the addition of the named metals a normal growth occurs on the nitrate media.

4. The pigment production is not dependent on the quantity of the mycelium produced nor on the age of the cultures. In some media, such as NH_4Cl , the same amounts of mycelium are formed as on nitrates, but are entirely colourless in the former case. Metals had no influence on the pigment formation. In normally pigmented cultures, only K and Mg or Ca, K and Mg, or Na, K and Mg were present. The addition of further metals caused no pigmentation in colourless cultures. Pigment production is apparently closely bound to the assimilation process and to some oxidation process.

5. Decreasing the concentrations of the solutions did not reestablish the capacity of synthesis of either of the vitamins.

6. Biotin can be completely replaced by aspartic acid or oleic acid on nitrate media.

References

1. Bortels, H.: Über die Bedeutung von Eisen, Zink und Kupfer für Mikroorganismen. — *Biochem. Zeitschr.* 181:301. 1927.
2. Fries, N.: Vitamin B_1 , vitamin B_6 and biotin as growth substances for some Ascomycetes. — *Nature* 152:105. 1943.
3. — The nutrition of fungi from the aspect of growth factor requirements. — *Trans. Brit. Myc. Soc.* 30:118. 1948.
4. Leonian, L. H. & Lilly, V. G.: Studies on the nutrition of fungi. I. Thiamin, its constituents and the source of nitrogen. — *Phytopathology* 28:531. 1938.
5. Metz, O.: Über Wachstum und Farbstoffbildung einiger Pilze unter dem Einfluss von Fe, Zn und Cu. — *Arch. Mikrobiol.* 1:197. 1930.
6. Perlman, D.: On the nutrition of *Memnoniella echinata* and *Stachybotrys atra*. — *Amer. Journ. Bot.* 35(1):36. 1948.
7. Potter, R. L. & Elvehjem, C. A.: Biotin and the metabolism of *Lactobacillus arabinosus*. — *Journ. Biol. Chem.* 172(2):531. 1948.
8. Rennerfelt, E.: Inverkan av tallkärnvedens fenolsubstanter på några blåtesvampars tillväxt jämte ett försök till kvantitativ mätning av blånadens intensitet. — *Medd. Statens Skogsforskningsinst.* 34:7. 1945.
9. Robbins, W. J. & Kavanagh, F.: Vitamin B_1 and the growth of certain fungi. — *Amer. Journ. Bot.* 25:229. 1938.
10. Schopfer, W. H.: *Plants and Vitamins*. — Waltham, Mass. U.S.A. 1943.
11. Steinberg, R. A.: Relation of accessory growth substances to heavy metals, including Mo, in the nutrition of *Aspergillus niger*. — *Journ. Agr. Res.* 52:439. 1936.
12. — Use of microorganisms to determine essentiality of minor elements. — *Soil Sci.* 60(2):185. 1945.
13. Winzler, R. J., Burk, D., & Du Vigneaud, V.: Biotin in fermentation, respiration, growth and nitrogen assimilation by yeast. — *Arch. Biochem.* 5:25. 1944.

The Translocation of Salts and Water through Wheat Roots

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A. Some fundamental principles

a. *The osmotic state*

Previous investigations (9—12) have shown that the translocation of salts through the roots is promoted by two groups of processes, (1) the active *absorption* from the medium together with the active accumulation in the

cells of the root tissue, and (2) the release or *exudation* of the accumulated salts in the xylem of the stele. The absorption is linked to that part of the aerobic respiration which is inhibited by small quantities of cyanide or azide, but it is less sensitive to inhibitors of glycolysis. The exudation proceeds independently of the aerobic respiration, but it is indirectly dependent on the accumulation mechanism charging the exuding cells with salts. The motive power of the absorption is the anion (or salt) respiration, a process probably identical with the cytochrome-cytochrome oxidase system, which enables the building up of a steady state of high concentration level. The exudation manifests the down-hill side of the steady state.

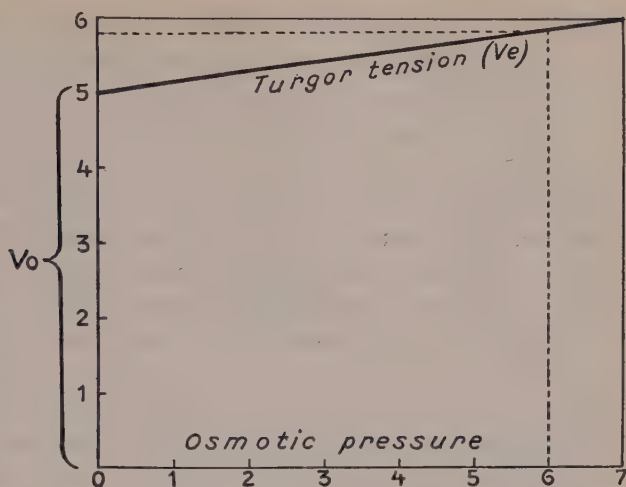
The translocation of salts is commonly accompanied by the translocation of water, but the latter can certainly be translocated without salts. The relation between the quantities of transported salts and water is determined by a number of factors. In a previous communication the osmotic fundamentals of the movement of solutions were discussed on the basis of the osmotic model of an indefinitely extensible cell. It was concluded that (1) exuded osmotic particles are accompanied by a quantity of water corresponding to the relation water : solutes in the exuding cells, (2) »extra water» is exuded if osmotic particles disappear in the cellular metabolism, and (3) the hydrostatic pressure exerted by the exudate is identical with the turgor pressure of the exuding cells. On these premises the results of the previous experiments regarding the bleeding of wheat roots were discussed (11, 12).

The majority of the cells of the root tissue, however, are capable only of a rather limited turgor extension. The quantity of water participating in the movements of osmotic particles in or out from the cell is limited by the dimensions of the plasmolyzed and the fully turgescient cell, determined by

$$V_e = V_t - V_0$$

where V_0 = the volume of the cell at the osmotic pressure P = zero, V_t = the volume of the fully turgescient cell, and V_e = the volume representing the elastic tension of the cell wall, the latter at osmotic equilibrium compensating the pressure P . P is here the net pressure (osmotic value of the cell minus osmotic value of the surroundings). As the true curve of elastic tension is not exactly known (it is probably a sort of convex curve) it is assumed, for the sake of simplicity, that V_e varies linearly with P (see fig. 1). It is also assumed that P varies linearly with the number n of osmotic particles in the cell, due reservation thus being made for van der Waals forces, hydration and other correction factors and cooperating processes (see below). Under these simplified conditions the gain or the loss of $1/n$ osmotic particles entails the uptake or exudation of a quantity of water corresponding to $1/n \cdot V_e$.

Fig. 1. Diagram of the relations between osmotic pressure, turgor tension, and volume of the cell. V_o = the volume of the cell in plasmolyzed stage. V_e = volume of the additional elastic stretching of the cell.



The degree of elastic tension of the cell wall thus determines the quantity of water moved together with the osmotic substances. Broadly speaking the »pumping capacity» of a cell is a function of V_e/V_o , if »pumping» means volume water (=a solution of exuded osmotic substances, or extra water). A further conclusion is the thesis that a cell without elasticity can »passively» exude very concentrated solutions, because the osmotic equilibrium is not influenced by a loss or gain in P . If not immediately removed from the contact with the exuding cell the exuded quantity of osmotic substance, however, exerts an osmotic suction on the former, finally resulting in an equalization of the outer and inner P -values. If the exuding cell is surrounded by neighbours the exudate can continue to suck water through the whole tissue until an average suction equilibrium is attained. Because in the case of cells with elastical turgor tension variations in P refer to the total volume of the cell (V_t), but the corresponding variations in the water content only to a fraction of this volume (V_e), the exudate in the moment of outflux is more concentrated than the cell contents. Owing to the fact that water, as a rule, moves faster than salts, however, an equalization sets in almost simultaneously with the process of exudation, resulting in a dilution to a value approximating that of the exuding cell (=»equiosmotic exudation») or, if water can be sucked through the tissue, below this value.

As a rule the exudation is canalized, *viz.* only certain cell contents are given off, e.g., primarily salts. If the osmotic power of the cell is exerted by sugar and salts and only salts are exuded this means a considerable rise in concentration of the latter, or generally in the relation $\frac{O+y}{y}$, if O =the sum of other osmotic substances in the cell and y =the exuded ones.

Applied to our present problem, the internal exudation from the root cortex to the stele, and only referring to the outflux of solutes, not extra water, the conditions favouring an osmotic equilibrium between tissue and sap will be realized if the outlets of the vessels are clogged. In a normal upward movement of the sap no osmotic equilibrium is attained but the concentration of the sap will be the resultant of a number of circumstances including the permeability to water, the dimensions of the vessels and the velocity of the sap. These adjacent circumstances are specific characteristics of the subjects of investigation, a fact which explains the fairly constant concentration of the ascending sap from roots grown under identical conditions (10, 11).

The elastic turgor tension of the parenchyma of the wheat root is probably rather below than above 20 %, but it is difficult to measure. Calculating with the figure 20 % and a movement of water slow enough to delay the osmotic equalization the salt solution exuded into the vessels can theoretically be expected to attain a maximum concentration five times higher than that of the cell sap. If 50 % of the osmotic power of the exuding cells is due to other substances, e.g., sugar, the maximum will be twice as high. But these theoretical maximum values are actually never observed. In wheat roots the sap is chiefly composed of a solution of potassium nitrate (10). The average concentration of nitrate in the tissue of normally fed roots is 40—80 millimols/litre and ten times that figure would result in a sap exerting an osmotic pressure of 20—40 atmospheres. Direct observations give far lower values. Even under extreme conditions — the blocking of the translocation by cyanide or N_2 — the exuded sap contains up to c. 50 millimols/litre, in fluoride up to c. 120 millimols/litre (p. 145). Normally the concentration remains at much more modest values.

Two circumstances are responsible for this result: (1) the rapid osmotic equalization between exuding tissue and exudate, mentioned above, and (2) the simultaneous exudation of »extra water», *viz.* water exuded owing to metabolic disappearance of osmotic substance in the exuding cells, e.g., the combustion of sugar and the proteinization of nitrate. The influence of the last named process has been extensively demonstrated and discussed in a previous paper (11). In wheat roots 40—50 % of the absorbed nitrate is converted into proteins and withdrawn from the osmotic balance. This means a liberation of a quantity of extra water corresponding to a dilution of the sap to its double volume, provided that the exuded water seeks its way to the stele instead of disappearing through the epidermis. Certain facts, indeed, support the assumption of the nitrate assimilation located in the interior of the tissues.

An important promotor of osmotic power is the respiration. Sugar is

conveyed from the leaves through the phloem and from here invades all living cells of the root. At complete combustion one molecule glucose produces six molecules of carbon dioxide. The corresponding sixfold rise in its osmotic power, no doubt, contributes to the turgor of the living cell, but the overwhelming part of the carbon dioxide escapes through the epidermis, thus adding very little extra water to the ascending sap (11). But it must be assumed, that the water osmotically carried by the descending stream of sugar is partly transferred to the ascending stream of mineral salts.

Some authors believe in certain »anomalous» components of the osmotic state of the living cell, *viz.* vital processes causing an influx of water, hypothetically producing an enhanced up-hill pressure wave working against the downhill outflow of water not held by static osmotic forces. One of those components is believed to be the electric charge of the membrane of the cells (20). Calculations of the gradients of electromotive power needed for an electro-osmotical translocation of water (see 10, p. 40), however, indicate that the gradients around 60 mv. observed at the surface of roots have a very limited power in this respect. The extra water moved according to the changes in osmotic power during the metabolic life of the cell, no doubt, is responsible for a certain fraction of the total osmotic value. Already the carbon dioxide plays the rôle of a »vital component» in the osmotic state of the cell. Of a metabolic nature are undoubtedly also the observed differences in osmotic values obtained in plasmolysis experiments with sugar and salt solutions (1). From the viewpoint of the theory of anion respiration a cell brought into contact with a salt solution and provided with the power of anion respiration must show a higher osmotic value than the same cell in contact with a solution of sugar, provided the sugar is not actively absorbed too. The anion respiration mechanism continuously pumps salts into the cell and thus continuously raises its osmotic value in relation to the imported ions. This fact was overlooked in the older plasmolysis experiments and is probably one of the reasons, why so varying results as to the permeability to salts (6) were obtained. Only a careful examination of the power of active accumulation of salts, sugar, or any other plasmolytical agent is able to reveal the true nature of the assumed »anomalous» components of the osmotic state. From the viewpoint of anion respiration and the theoretical discussions presented in this and earlier papers of the author no living cell shows an osmotic state of the type of a physical model. It is on the contrary always the question of a steady state and a fraction of the observed osmotic power is regularly carried by metabolic processes of any kind. All manipulations introduced for the purpose of measuring the osmotic power of the cell simultaneously cause

a disturbance of the metabolism. These create the only real »anomalous» component of the osmotic state of the living cell.

b. *The anion respiration*

The exudation of salts in the xylem of the stele represents the down-hill side of a steady state maintaining the concentration level of nutrient salts characteristic of roots. The exudation means a lowering of the osmotic potential. The active absorption of salts from the medium forms the up-hill side of the steady state, working in the direction of rising the osmotic potential. It was previously shown (11) that this process is not restricted to the surface layer of the root only, but occupies all cells of the tissue serving as a pathway from the medium to the stele. The motive power of the salt accumulation and active salt transport from cell to cell is the anion respiration, according to a theory developed by the author, identical with the cytochrome-cytochromeoxidase system. The process is inhibited by small quantities of cyanide and azide. It is checked by carbon monoxide in the dark and this inhibition is reversed in the light (18). The quantitative relations between the anion respiration and the absorption of anions are illustrated by experiments with gradually increasing inhibition: the anion respiration and the anion absorption decrease in the same relation (Q_{an}/O_2 = practically constant; see 14). The anion respiration is superposed on the ground respiration which is characterized by its low sensitivity to cyanide. In the lower parts of the roots (0—30 mm. from the tip) a »third respiration» is added, the functions of which are perhaps in some way linked to the growth processes (14).

According to the theory of anion respiration, anions move in the opposite direction to that of the electrons taken over by the cytochrome system from a hydrogen donor (a reduced dehydrogenase) and transferred through the different cytochromes and cytochromeoxidase to the molecular oxygen. The ionized oxygen then reacts with the hydrogen ions forming water. This is the main energy-liberating process of the respiration.

The theory postulates that the single members of the cytochrome system are attached to an organized structure in such a way that an »electron ladder» is formed (see fig. 2). Anions will then be transported along the ladder, in the opposite direction to the transference of electrons between the active iron atoms. The process can also be figured as an electrical current generated by the oxidation process, the internal branch of the circuit being the path of electrons, the external branch the movement of anions. Cations, of course, migrate in the opposite direction to that of the anions; this demand will be fully satisfied by the movement of H-ions from

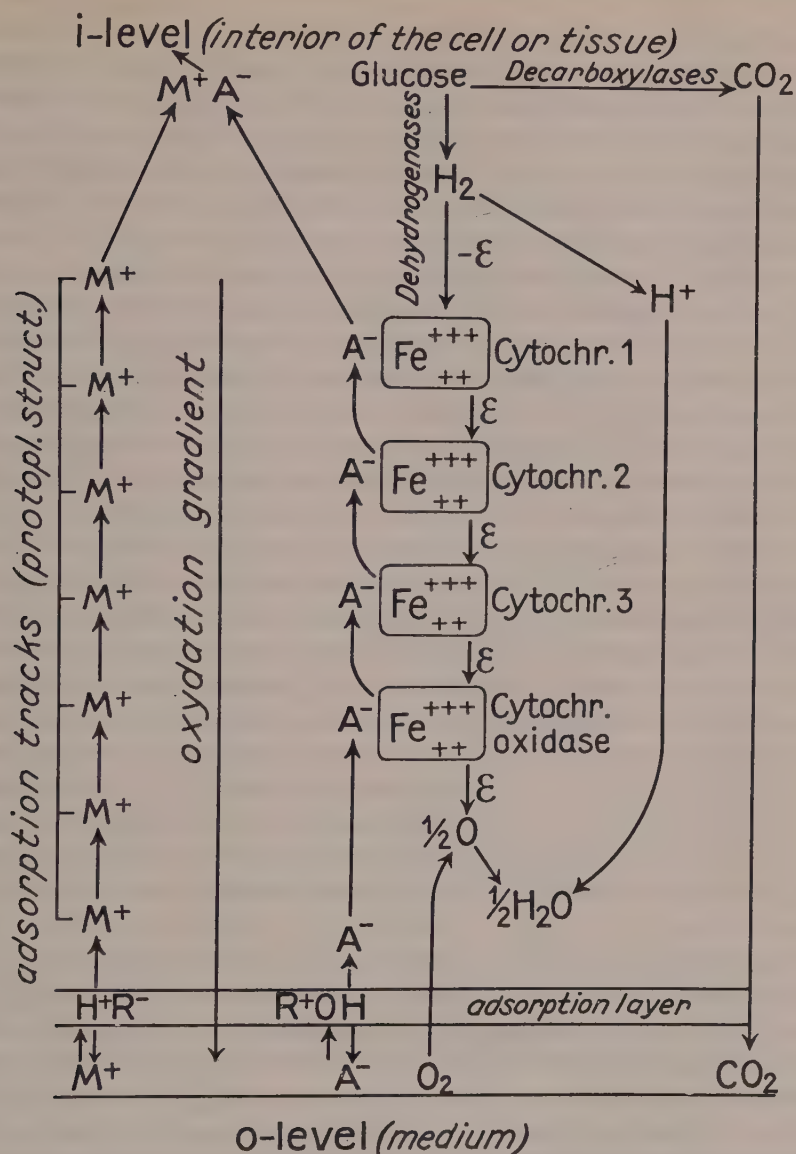


Fig. 2. Diagram of the theory of anion respiration. Explanation in the text.

the place of ionization (=towards the positive end of the ladder) to the place of deionization (=at the negative end).

The anions arriving at the positive end of the electron ladder are combined with cations from the surroundings in relation to the activity or frequency of the latter. Owing to the predominant acid dissociation of the components

of the protoplasm metallic cations are everywhere abundantly adsorbed as a consequence of which the liberated anions appear as salts of potassium, calcium etc. The fact that the cell sap almost invariably has a lower pH than the medium, or the protoplasm, is probably due to the liberation of a part of the anions as acids. This is primarily the case with the anions of organic acids competing with the neutral salt anions as objects of transpiration of the anion respiration (see 14).

Still undecided is the localization of the cytochrome-cytochromeoxidase system in the cell. Its firm attachment to the protoplasmic structure has been established. In respect to the enzymes participating in the carbohydrate metabolism of yeast cells some facts indicate a localization in or near the surface or in the interior of the cell (16). If the electron ladders of the cytochrome-cytochromeoxidase system are built in perpendicularly to the surface of a membrane separating two levels of accumulation, e.g., the medium and the bulk of the protoplasm, or the protoplasm and the cell sap, salts will be actively accumulated inside the membrane, provided that oxygen comes from the outside and the donated hydrogen from the inside. The salts will generally be transported according to the polarity of the enzyme system (see fig. 2). But a polar transportation can be figured also as the statistical result of a more irregular distribution of elementary structural particles, carrying electron ladders, between two oxidation levels. The intensity of electron transfer through the elementary particles will in this case be directly related to the magnitude of the component perpendicular to the oxidation-reduction gradient. As the limiting action of the oxygen appears only at very low tension the influence of the oxygen gradient, however, seems to be less pronounced than that of the structural polarity of the enzyme system itself.

As mentioned above previous investigations support the conclusion that the anion respiration is not restricted to the surface of the root only but operates in the bulk of the tissues. Two possibilities may be considered here. If the tissues (primarily the cortex) are built as a »symplast» salts may flow through the plasmodesms and have to pass cell walls only at the places of absorption and internal exudation. The places of absorption and exudation are in this case wide apart and an anion respiration mechanism operating in the bulk of the tissue would have the character of an accelerator. If, on the other hand, the single cells of the tissue behave more as »elementary organisms» the salts will have to pass through a large number of cell walls functioning as an intermediate salt solution. Each single cell would under these circumstances maintain a steady state manifested as a dynamic balance between the anion respiration (up-hill side) and a passive tendency to loose salts (down-hill side). The idea of the cell walls participating in

the translocation of solutes was originally advanced by Sachs and later elaborated by Crafts, Strugger and others. More than a function as a passive intermediate cannot be attributed to the thin cell walls of the root tissue. Only the living protoplasm is endowed with the power of active translocation and accumulation, a process always involving supply of energy. Whereas nothing can be said at present about the function of plasmodesms as pathways of salt transport anatomical-physiological experience speaks in favour of accumulation and exudation processes simultaneously going on in the single cells and these co-operating in the translocation of salts from the surface of the root to the stele.

Some facts concerning the different behaviour of the anatomical layers of the root may be recapitulated from previous investigations. The epidermis is primarily endowed with salt absorption. It allows an abundant exchange of cations with the medium but unwillingly releases anions of neutral salts, in spite of its own electro-negative charge. Surprisingly many organic substances are given off from the normally functioning epidermis, *e.g.*, sugar, nucleotides, etc. An opposite behaviour characterizes the parenchyma of the stele surrounding the xylem vessels. In the following pages this tissue is called the »*vascular epithelium*». The vascular epithelium readily gives off both cations and anions of neutral salts but no organic substances. It acts most satisfactorily as a tissue of salt excretion. From another viewpoint it may be said that the vascular epithelium behaves as the inside of the epidermis layer, but it is, on the other hand, not known if the former develops any appreciable anion respiration, as the cortex undoubtedly does. The outside of the epidermis and the inside of the vascular epithelium are facing different media but this fact does not cover the whole problem. The ontogenetical determination of the epidermis as a barrier against losses of absorbed salts is probably a factor of great physiological significance. Experiments with roots deprived of their epidermis (*cf.* 4), if extended to nutrition problems, would be of great interest here.

Contrary to the outside of the epidermis all cellular boundaries facing living neighbours, *e.g.*, the protoplasts of the cortex, must be assumed as being comparatively »permeable» to salts. Observations as to the distribution of salts in wheat roots give the impression of a rapid flowing of salts inside the epidermis. It is difficult to ascertain, however, if the salts are traversing the protoplasmic membranes by means of diffusion or *via* rapid ion exchange processes. Studies in the absorption of salts through the boundary between a tissue and a solution (experiments with roots or slices of storage tissue; see 9, 11, 14, 17) convey the idea that the primary stage of absorption is an ion exchange occurring in the protoplasmic membranes. No principal objection can be raised against the assumption that this non-metabolical ion

exchange also penetrates to deeper layers of the tissue. In the case of cations this is an established fact. It has also been shown that this non-metabolic exchange of cations proceeds with considerable speed (15) and has a purely non-polar character. The end result is much the same as that commonly called »permeability», but exchange processes are proceeding far more rapidly than simple diffusion.

The exchange of anions between the medium and a root is usually hampered by the high electro-negative potential of the latter (see 11, 14). Inside a tissue the membrane potentials are probably very close to zero, as a consequence of which the exchange of anions will rise to the same degree as that of the cations. Similar conditions are probably prevailing at the surface of the vascular epithelium. Ion exchange seemingly offers an excellent opportunity of »canalized leakage», *i.e.*, a permeability restricted to salt ions, because entry or exit of ions is restricted to the ionized points of the protoplasmic membrane, whereas non-dissociated compounds have to penetrate the ground substance of the latter. On the basis of such a dualism in the physiological morphology of the protoplasmic membrane a very restricted permeability to non-dissociated substances can be combined with a rapid penetration of ions, and *vice versa*, according to the special chemical and physico-chemical properties of the membrane.

B. The aim of the present investigation

The exposition of the fundamentals of the osmotic and metabolic processes maintaining the translocation of salts through the roots given above is based on previous investigations, published between 1938 and 1949 (8—15). As to the question of the anatomical substrate of translocation it was assumed that salts and extra water are withdrawn from the vascular epithelium in the lower parts of the root and emptied into the open vessels. According to the hydrostatic pressure the sap seeks its way through the vessels and eventually is forced through the hydathodes of the leaves. At simultaneously occurring transpiration the volume and speed of the ascending sap is increased with pure water sucked through the living tissues. According to this picture of the sap stream the lower parts of the vessels acted as the container of an osmometer and the continuation of the conducting system as the tube of the osmometer. The motive power of the whole system was attributed to the anion respiration.

A closer examination of the site of active salt absorption now revealed the fact that not only the tip zone but the whole epidermis of young wheat roots up to a height of at least 100 mm. uniformly absorb salts from the

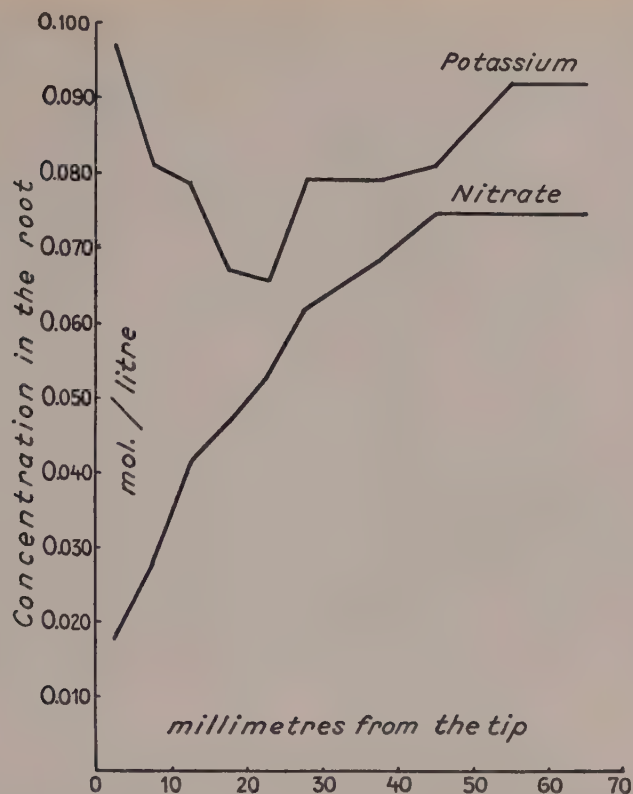


Fig. 3. The concentration (per unit root volume) of nitrate and potassium in the wheat root (see 11, p. 12). K and NO_3 vary approximately parallel in the basal part of the root (from c. 30 mm. upwards). In the tip zone (c. 0—30 mm.) these ions behave opposite to one another. In all zones anions are actively absorbed from the medium (14). In the basal part of the root practically all absorbed potassium nitrate is exuded in the stelar vessels and disappears with the ascending sap stream. In the tip zone about 50 % of the absorbed nitrate is reduced and «proteinized» and only the rest is conveyed to the stele. The nitrate assimilation creates a surplus of cations (potassium) which is probably partly balanced by organic acids. As a considerable part of the «extra water» of the ascending sap comes from the tip zone the supernumerary potassium ions which are under certain conditions observed in the sap (together with HCO_3) are probably withdrawn together with the water.

medium. If the supply from the medium ceases the stored quantities of salts slowly disappear, at first in the tip zone, but later on also in higher levels (fig. 3). It was supposed that part of the latter quantities are moved downwards through the cortex, but this question needs further elucidation. If the root tips are cut off and the vessels consequently opened the upper end of the root still continues to bleed, but at a speed the more reduced the larger the size of the removed endpiece. The possibility was discussed (12) that part of the ascending sap stream moves through the cortex, at least in

the lower regions of the root. But also this question needs further experimental investigation. Recently the metabolic side of the internal exudation of sap was taken under consideration (13). A series of experiments with various biochemical inhibitors (iodine acetic acid, fluoride, cyanide etc.) revealed the fact that the sap exudation proceeds fairly independently of the anion respiration, the latter only providing the material of exudation. From the remarkably strong inhibiting effect of fluoride on the bleeding it was concluded that the sap exudation is conducted by glycolytical processes. In these experiments, however, only the total quantity of bleeding sap was measured, not the separate exudation of salts and extra water, and the possibility of an opposite reaction of these two elementary processes was still left open. Even here further investigations are needed. In the present communication the whole problem has been reexamined.

An important part of the experiments was devoted to an examination of the degree of contribution of the different zones of the root to the total sap exudation. Simultaneously the participation of the cortex, the stele, and the open vessels, in the process was experimentally elucidated. The anatomy of the root was studied on living and fixed material and with the aid of vital staining. Finally the exudations of salts and of water were separately investigated.

C. Experimental methods

As in previous experiments the material was 2—3 weeks-old seedlings of spring wheat (for the composition of the nutrient solution, see 10, p. 4; for the culture in photothermostats, see 7).

The sap exudation was studied on decapitated seedlings (see 11, p. 50, fig. 11) or, mostly and preferably, on single roots. These were cut off below the level of visible secondary roots (70—100 mm.; the secondary roots were initiated c. 30—40 mm. from the tip) and introduced into the slightly widened end of a 0.4 mm. capillary tube (the diameter of the roots is approximately 0.5 mm.). A pincette with rubber points was used for this procedure. Numerous control experiments have shown that the joint between the root and the conically widened capillary tube was satisfactorily tight. No water was capillarily sucked from this junction. The bleeding was not affected by the slight pressure exerted on the cortex in introducing the root in the tube.

The capillary tube was held by a horizontal arm adjustable by means of rack and pinion (see fig. 4). Another similarly adjustable arm carried a small glass basin containing the medium (mostly distilled water), in which the roots were submerged to a point 1—2 mm. below the end of the capillary tube. The basin was 50—80 mm. high and held 20—50 ml. It was

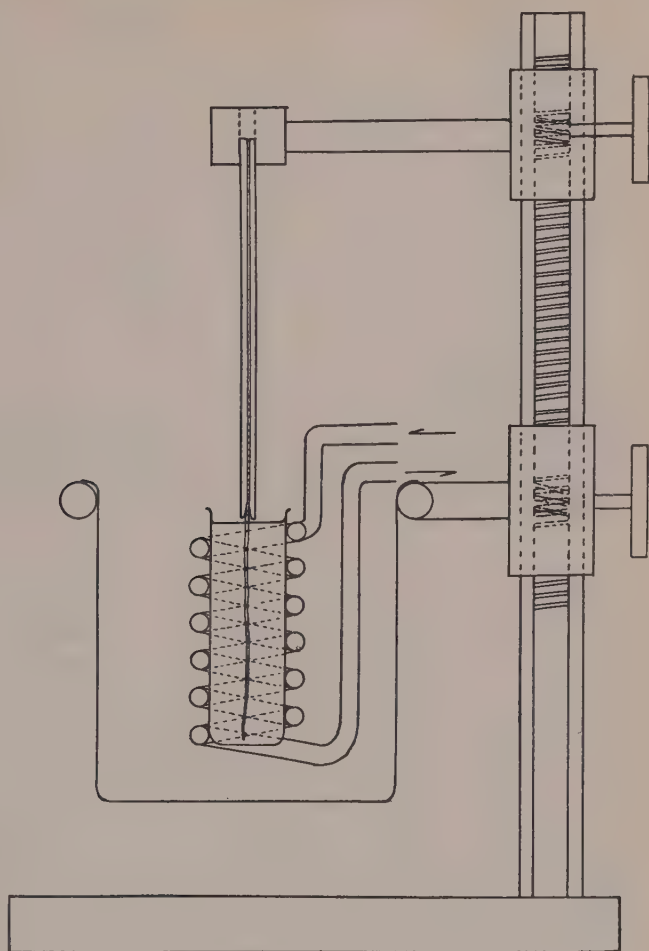


Fig. 4. Scheme of the device for measurements of the bleeding of single roots. Explanation in the text.

surrounded by a glass spiral connected by means of an electric pump to a water bath of constant temperature. The glass spiral was enclosed in a large glass beaker filled with water. This device ensured measurements of the bleeding at constant temperature (mostly 24°C). By means of the rack and pinion arrangement the root could be rapidly hoisted up or down and the solution in the basin could be changed without disturbing the measurements of the rise of the capillary sap pillar in the tube.

The horizontal microscope used for the measurements was firmly attached to the stand and could be moved round a vertical axis. If the bleeding from both ends of a root segment had to be measured these were introduced in two separate tubes and the root carefully bent 180° so that the tubes could be parallelly attached to the stand (see fig. 5).

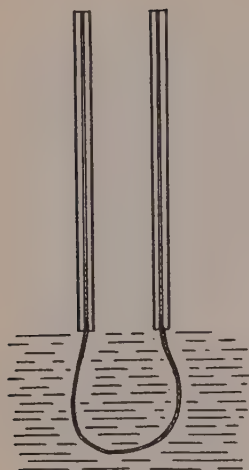


Fig. 5. *Measuring arrangement for the determination of the bleeding from both ends of a basal piece of a root.*

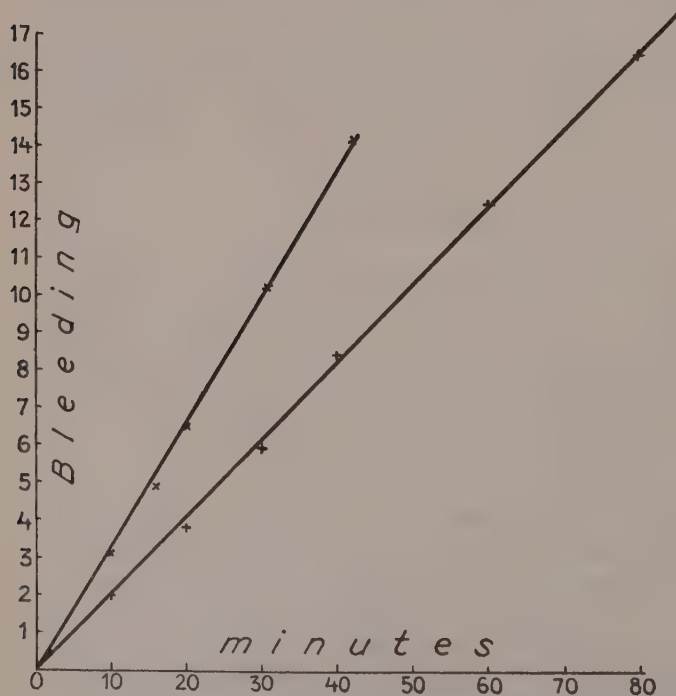


Fig. 6. *The normal time course of the bleeding from two roots. The medium is distilled water.*

The time course of the bleeding of a root surrounded by distilled water was fairly constant during a period of 1—2 hours, or even longer (fig. 6). This circumstance facilitated investigations of the effect of various substances. The average bleeding of a single root taken from a nutrient solution and transferred to distilled water amounted to 2.64 mm.³ per hour at 24° C.

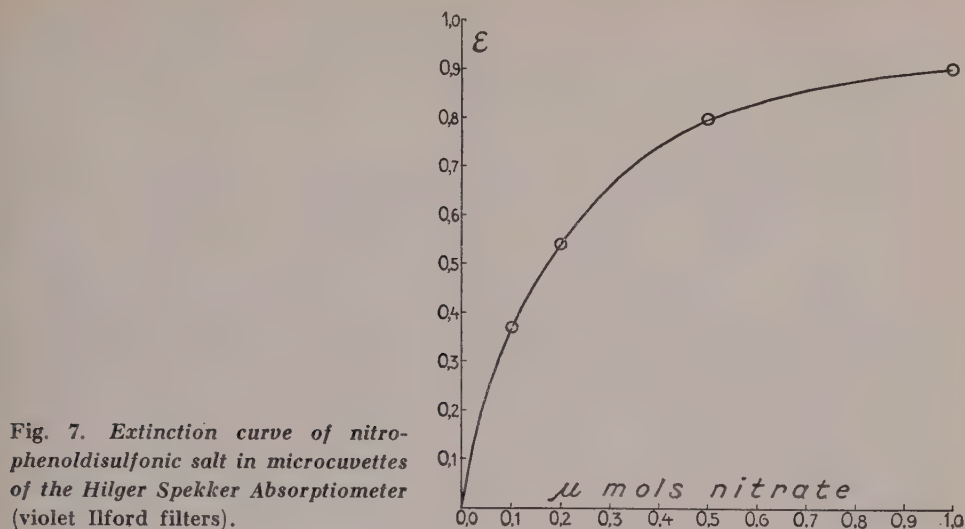


Fig. 7. Extinction curve of nitrophenoldisulfonic salt in microcuvettes of the Hilger Spekker Absorptiometer (violet Ilford filters).

In special experiments the cortex was squeezed and intersected, or the stele intersected at intact cortex, or the vessels clogged at one end of a root segment. These encroachments will be described later on.

The exudation of nitrate in the bleeding sap was followed by chemical analyses (the phenoldisulfonic acid method). With the aid of microcuvettes measuring 0.5 cc. and a Hilger Spekker absorptiometer (violet filters) nitrate quantities from c. 0.025 to 1.0 μ mol could be determined, corresponding to a few mm.³ bleeding sap. As a rule two or four bleeding tubes were run parallelly. The sap, the volume of which was calculated from the microscopic measurements, was transferred to a 20 ml. glass dish and evaporated on a water bath. Treatment with H_2O_2 (see 3) could be omitted because of the absence of organic substances in the sap. After cooling 10 drops of phenoldisulfonic acid were added from a standard pipette and carefully distributed on the walls of the dish by means of a glass rod. After 5—10 minutes standing 2 drops of distilled water and 28 drops of 50 % NH_3 were added. The colorimetric determination was made immediately after the cooling of the sample. Its volume was c. 2 ml., enough for duplicate determinations. Owing to the convex absorption curve of the nitrophenoldisulfonic salt (see fig. 7) good determinations were restricted to the region 0.05—0.5 μ mol NO_3 .

The same method was also used for determination of the nitrate exuded from roots submerged in solutions. Twenty to thirty root segments were submerged in 10—15 cc. solution. After removing the roots the solution was evaporated on a water bath. If necessary decarbonization with H_2O_2

(3) was performed before treating the residues with phenoldisulfonic acid. In some experiments the bleeding sap of a bundle of roots was collected in cotton wool (cf. 11) and washed off in distilled water before the analysis.

D. Presentation and discussion of the experiments

a. *The power of bleeding in different zones of the root*

In a number of experiments the root was inserted in the capillary tube, the bleeding measured, then a shorter or longer piece of the tip cut off and the bleeding again determined. Measurements were made at intervals of 5 minutes and continued for at least 30 minutes after each treatment. Owing to the constancy of the exudation good averages could be calculated of the bleeding per minute.

The cutting off of a piece of the tip suddenly changed the speed of the bleeding stream and the process then continued almost constantly at the reduced speed. In table 1 and following tables only relative values are given, the bleeding of the intact root at the beginning of the experiment was taken as 100. Removal of the tip (1—3 mm.) on an average stimulated the bleeding (see table 1). Removing larger pieces of the root end retarded the bleeding in relation to the length of the removed zone.

A few experiments were performed to determine whether the method of cutting (by scissors, or by razor blade) had any effect, or if it made any difference if the ends were not cut off but just killed by alcohol (see 12). It was concluded that cutting by razor blade gives somewhat lower values of the bleeding from the remaining part of the root than cutting by means of scissors. The result was attributed to a certain backflow through the opened vessels. The scissors partly squeeze the vessels and reduce the dimensions of the free openings. Treatment with alcohol leaves the vessels closed. The bleeding from the basal end was in this case higher than after cutting off end pieces of the same length (see 12, p. 143, fig. 6). All these experiments support qualitatively the idea of an exudation of most of the sap into the open vessels. The slight stimulation of the bleeding after removing only the growing tip was attributed to the falling away of a branch of the sap normally conveyed to the meristem.

If the reduction of the bleeding of the decapitated roots is caused by a back-flow through the lower end which is surrounded by the solution the escaping quantities can be saved by introducing the lower end in a second capillary tube (fig. 5). Both tubes together will then give the total sap exudation of the remaining basal segment of the root. Furthermore, if the removed apical piece is introduced into a third tube, it is possible to calculate

Table 1. *The bleeding from decapitated seedlings, after cutting off the tip zones of the roots at varying height. Each value is the average of 5—16 single experiments. Relative values (the bleeding of the intact plants=100). Each plant carried 3—5 roots of a length up to 100 mm. Temp. 23° C.*

Length of the removed root tips in mm.	1—3	3—5	10—15	25—30	40—50
Relative bleeding	110.4	89.0	59.0	33.0	26.5

Table 2. *Experiments with single roots. Bleeding of the intact root, the removed end piece, and the upper and lower ends of the remaining basal piece. Each value is the average of 3—6 single experiments. Bleeding of the intact root at the beginning of the experiment=100. Temperature 23° C.*

Length of the removed end piece in mm	Bleeding of the tip zone	Bleeding of the rest of the root (50—54 mm.)		Sum of 1, 2, 3
		Upper end	Lower end	
	1.	2.	3.	
15	5.6	73.6	12.4	91.6
20	11.1	56.5	24.9	87.5
25	18.0	42.0	27.5	87.5
30	29.3	41.8	31.4	102.5

the percentual contribution of the two root pieces to the total bleeding of the intact root. A series of such experiments is computed in table 2.

In comparison to table 1 the bleeding values in table 2 referring to the upper end of the basal piece are about 20 % higher, but this difference is probably due to the fact that plants, not single roots, were used in the first-mentioned series. It will be shown in the following that the resistance to the translocation is higher in the stalk (and leaves) than in the roots. The losses from back-flow through the opened vessels must accordingly be higher. Tables 1 and 2 both show a decrease in the bleeding from the upper end of the root with an increase in the length of the removed apical piece. The bleeding did not come to a stop even when 40 mm. of the apical end were removed. Secondary roots if present, of course, introduce an accelerating factor.

As expected the removed tip zone shows an intensity of bleeding increasing with its length. But even the participation of a 30 mm. long apical piece to the total bleeding of the root remains surprisingly low, c. 30 %, in spite of the fact that the tip is the metabolically most active part. Also the root hairs are most abundantly present in this zone.

The sum of the separate bleedings of (1) the apical end, (2) the upper and (3) the lower ends of the basal piece of the root is generally somewhat

lower than the bleeding of the intact root, but table 2, nevertheless, convincingly illustrates the fact that the gap between the bleeding of the end zone and of the upper end of the remaining basal piece is filled by the exudation from the lower end of the latter. The results thus corroborate the assumption of a back-flow through the opened vessels and the previous idea of the xylem of the stele as the main conducting tissue. If the lower end of the basal piece of the root is left open, as in the experiments quoted in table 1, the sap exuded in the vessels flows in both directions, upwards and downwards, but only the first named stream is really measured. In the experiments with tubes on both ends of the basal piece (table 2) the back-flow is recorded. But also other possibilities are to be taken into consideration, e.g., the existence of a descending stream in the cortex, the direction of which would then be reversed in the tip zone. Before the experimental answer to this question is presented it has to be shown anatomically that salts can move from the cortex into the stele also in higher levels.

A certain amount of bleeding from the lower end of the stele is probably caused by the descending stream of organic compounds, chiefly sugar, through the phloem. A root of 50 mm. length consumes c. 0.02—0.03 μ mol glucose per hour. Assuming a concentration of 0.1 mol/lit. in the phloem sap this would result in an exudation of 0.02—0.03 mm./1 min. in the capillary tube, corresponding to 15—25 % of the observed bleeding from the lower end of a basal root piece. Owing to a partial clogging of the sieve tubes the real exudation will probably be lower.

b. The physiological anatomy of the wheat root

The length of the growing zone of the tip is 4—5 mm. (in secondary roots only 1.5 mm.). The maximum of elongation proceeds at c. 2 mm. from the vegetation point (v.p.), but the differentiation of central tissue, designed as the stele, is already visible (fig. 8 A), including the central vessel. On transverse sections a single layer of medium-sized cells around the smaller cells of the prostele probably marks the beginning endodermis. The vessels of the stele (seven in the present material) cannot be distinguished below a height of 15 mm. from v.p. on transverse sections, but on longitudinal optical sections of living roots reticulate vessels are clearly seen already at 6 mm. from v.p.

The diameter of the roots of wheat seedlings is c. 0.5 mm. The central vessel (sometimes two vessels can be distinguished; see fig. 9 D) has a diameter of 0.04 mm. in the zone of maximal growth (2—2.5 mm. from the apex) and 0.07 mm. at a height of 50 mm. The diameter of the stele is

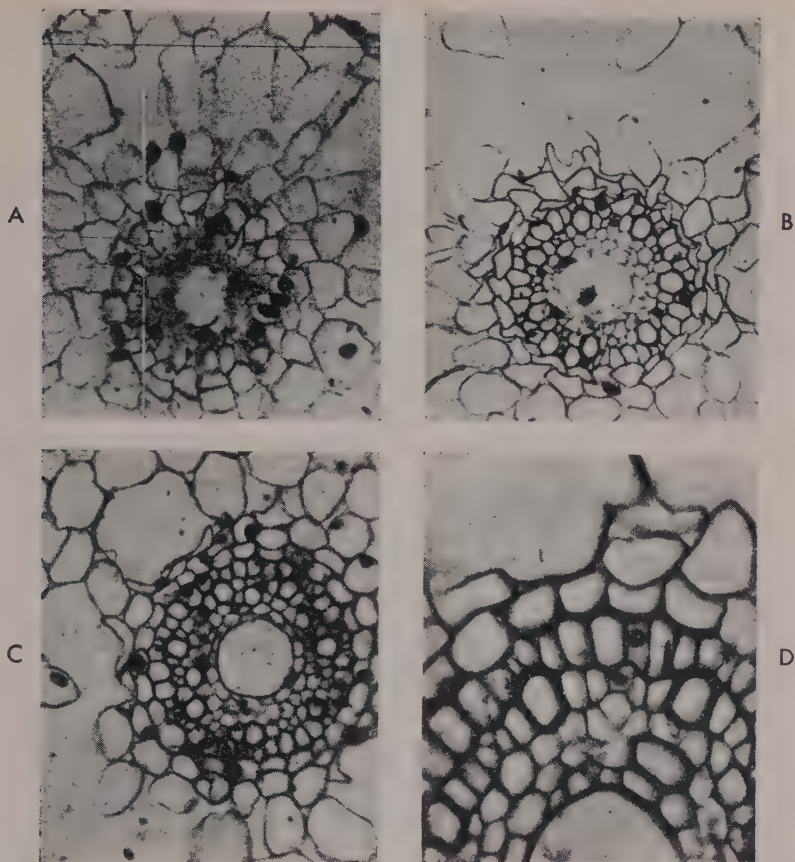


Fig. 8. *Transverse sections of a wheat root, fixed and stained in safranin-gentianaviolet.* A. 2.5 mm. from the tip ($\times 250$). B. 22.5 mm. from the tip ($\times 250$). C. 37.5 mm. from the tip ($\times 250$). D. 57.5 mm. from the tip ($\times 500$). — The dark corpuscles are nuclei.

0.15 mm. in the zone of elongation and 0.20 mm. at a height of 50 mm. The areas of the transverse sections are the following (in square mm.):

	Distance from v.p.	
	2.5 mm.	50 mm.
Total area	0.196	0.196
Stele	0.020	0.030
Central vessel	0.001	0.004
Small vessels	not developed	0.0008
Cortex	0.175	0.163

The transverse section of the cortex is built up by 100—150 cells partly separated by intercellular channels, each cell, on an average, showing the

same area as the central vessel (the inner cells are larger, the more peripheral ones smaller; see fig. 8). The epidermis is composed of 50—60 cells with a cross section considerably smaller than that of the cortex.

The roots used for the anatomical investigation had an endodermal cylinder composed of 25—30, on an average 27 cells at a height of 37—38 mm. Of these 7 or c. 25 % (sometimes more) are not lignified passage cells. The U-shaped thickenings are not fully developed at a height of 20—25 mm., but an endodermal layer is clearly distinguished at that height (see fig. 9 C, D of a living root at a height of 20 mm.). Up to a height of 30 mm. the endodermal cells are squeezed and deformed in roots treated with fixing agents (see fig. 8 B). Between 25 and 30 mm. the inner tangential walls start the thickening process and at 35—40 mm. also the radial walls are partly lignified. At a height of 40—50 mm. the U-shaped thickenings are fully developed (fig. 8 D).

The tissues of the stele are composed by narrow cells without intercellulars. Only the vessels are clearly differentiated in the lower part of the root. At a height of 20 mm. the phloem is distinguished as groups of narrow cells between the xylem vessels (see fig. 9 C), but the rest of the tissue remains parenchymal. Numerous nuclei appear in fixed and stained sections, a circumstance suggesting an abundance of protoplasm in the cells. The pericycle which is forming a single layer outside of the vessels starts lignification at a height of c. 40 mm. This layer is considerably more resistant to squeezing than the young endodermis.

From the viewpoint of sap movement there is no anatomical hindrance to a comparatively free passage between cortex and stele up to a height of c. 25—30 mm. This fact is probably important for the ready access to the stream of sugar descending through the phloem. At higher levels this tissue is more efficiently screened off from the communication with peripheral tissues than the xylem vessels are. Judging from anatomical facts the passage cells of the endodermis primarily serve as the entries to the vessels facing the surrounding cortex (fig. 8 D). If the vessels act as the chief collectors and conductors of the salt stream through the cortex it is well provided anatomically for an efficient supply also in higher levels of the wheat root.

As to the qualities of the cortex in regard to the translocation in a longitudinal direction it should be noticed that the cells, in addition to their large volume (see above) have an average length of nearly 0.3 mm. and are firmly attached to one another at the transverse walls. Also the conducting living elements of the stele are lengthy cells. The same is the case with the endodermis (see fig. 11 B). Anatomically the resistance to movements of solutes in a longitudinal direction must be predicted as low. But

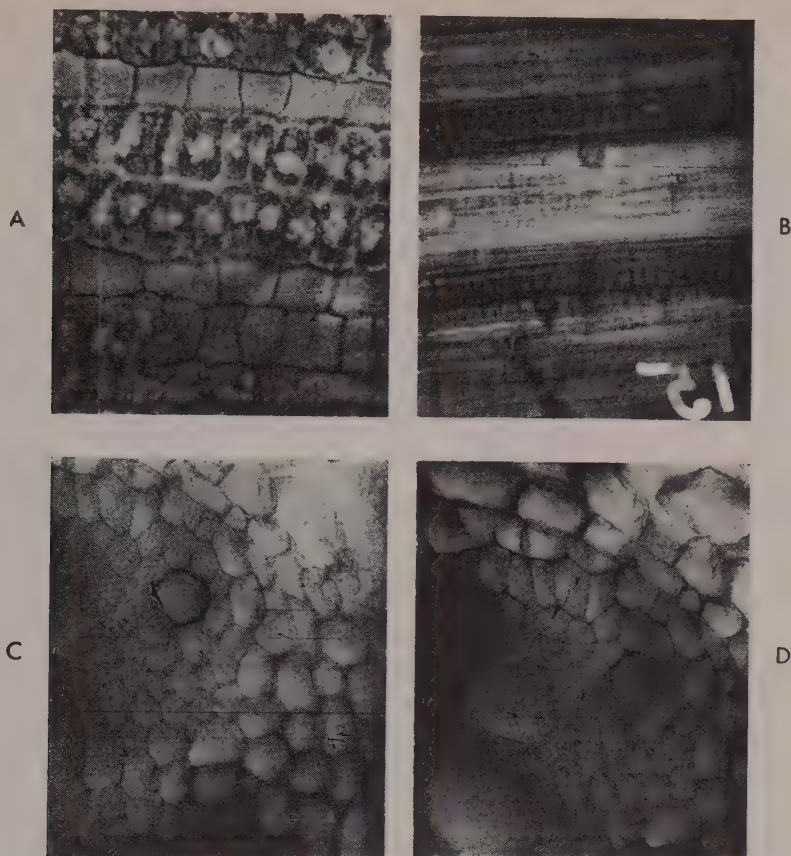


Fig. 9. Microphotographs from intact living roots (A, B) or transverse sections from living roots, all stained by neutral red (10 mg./l.). A. Accumulation of the dye in the vacuoles of the stretching zone (c. 3 mm. from the tip). B. Optical section of a root 15 mm. from the tip, showing the stele (reticulate vessels and long cells of the stelar parenchyma or endodermis). C and D. Sections 20 mm. from the tip, showing dark stained vessels, vascular epithelium, central vessel, phloem, pericycle, and endodermis (cf. fig. 8 B and C).

also the translocation in a radial direction, viz. from the cortex to the stele, is facilitated by the large diameter of the cells and the large contact areas. The intercellular channels do not appreciably encroach upon these contacts.

c. Experiments on vital staining

A solution of 1 mg. neutral red in 100 ml. water was comparatively harmless to the roots (see 5). The dye was rapidly absorbed and accumulated

in the vacuoles of the zone of stretching, whereas the protoplasm remained stainless (fig. 9 A; this is a criterion of its healthy state).

After two days in a solution, from which the dye had completely disappeared, the parenchymal elements of the apical part of the root remained unstained, whereas the vessels appeared dark red down to c. 6 mm. from the v.p. In the more basal parts the dye was absorbed also by the endodermal cells and the cells of the pericycle surrounding the vessels (fig. 9 B). Only those parts of the roots which stood in immediate contact with the solution absorbed larger quantities of the dye, but a very weak colouration appeared in some cortex cells above the level of the solution. The dyestuff was apparently absorbed through the epidermis, passed through the cortex cells preferably in a radial direction, and from here straight into the stele. This behaviour was observed at all levels, even in the tip zone. These observations are consistent with recent findings (14) of a fairly uniform absorption quality of the epidermis up to a height of at least 100 mm. They demonstrate the rapid translocation in the radial direction of the root tissues but are inconsistent with the idea of a more prominent translocation in longitudinal direction of the cortex or endodermis, especially with the existence of an ascending stream of solutes through the cortex.

The very characteristic vital staining of the vacuoles in the zone of stretching was a transitory phenomenon that disappeared after the exhaustion of the dyestuff in the medium. The dye apparently moved from these vacuoles in the cortex cells to the stele and was finally fixed in the lignified walls of the vessels. The whole process of translocation took less than 30 minutes. It was observed that the absorption was more intensive and began earlier in somewhat older parts of the stele, about 10 mm. from the tip. This fact is probably due to a more rapidly ingoing stream on this level. A curious differentiation of the transitory staining of the vacuoles was observed. Rows of stained cells alternated with rows of colourless ones (fig. 9 A), despite the fact that no anatomical differentiation between cells in one and the same histogen could be observed. This phenomenon remains unexplained, but it may perhaps be presented as an argument against exaggerated ideas of symplastic behaviour of anatomically uniform tissues (see p. 110). The duties of the cortex as a region of transition were also seen from the fact that the living tissues of the stele remain coloured long after the exhaustion of the medium and the decolorization of the cortex. The cortex cells were rapidly emptied in favour of the parenchyma of the stele. Finally, also the vascular epithelium is drained into the vessels.

The vital staining thus visualizes the pathways of translocation of mineral solutes, because these are also absorbed by the epidermis and exuded into

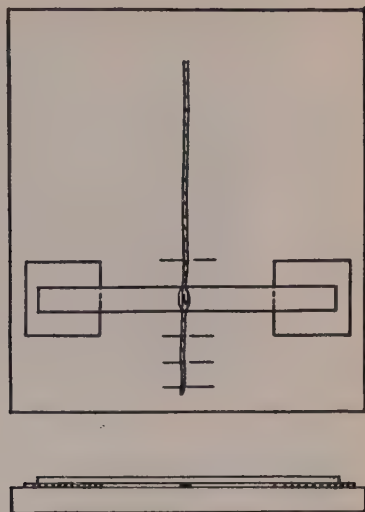


Fig. 10. Scheme of the device for squeezing and cutting of the cortex. Explanation in the text.

the vessels. Under the conditions of the experiments a certain amount of transpiration was added to the active processes.

d. *The rôle of the cortex and the stele in the generation of the ascending sap stream*

In order to elucidate this question a number of microsurgical experiments were performed. The thinness of the root impedes the accomplishment of a complete ringing of the tissue outside of the stele, but if the root is gently squeezed between two glass plates a considerable portion of the cortex can be intersected without injuring the stele.

On a perspex plate two microscopic cover glasses (0.15—0.18 mm.) were placed 30 mm. apart (fig. 10). A number of parallel lines were drawn on the perspex plate in order to facilitate the squeezing of the root at a certain distance from the tip. The plate was wetted by a few drops of water, the root placed perpendicular to the lines and finally squeezed by means of a 10 mm. wide and 60 mm. long strip of glass gently pressed against the cover glasses. Only the cortex is squeezed under these circumstances, its cell rows are spread apart, but the stele remains intact.

The bleeding was determined during a period before the squeezing and the observations continued after the root had been carefully returned to the capillary tube. These experiments showed the influence of squeezing alone.

In other experiments the squeezed cortex was intersected by means of two sharp knives applied from both sides and controlled through a

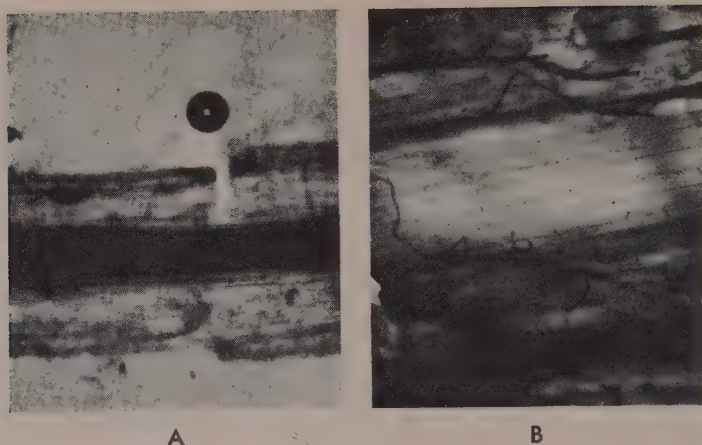


Fig. 11. *Microphotographs of living roots.* A. Squeezed and intersected. The cortex is nearly interrupted by the two cuts, but the stele intact. Bleeding still c. 70 % of that of the intact root. B. Squeezed. The surface of the stele, probably the endodermis, showing lengthy cells without intercellulars.

magnifying lens. Also in this case the stele can be protected from injury if care is taken to avoid breaking or bending of it in returning the root to the capillary tube.

In other experiments the stele was intersected and the squeezed cortex left untouched. In all experiments the squeezed or intersected part of the root was scrutinized under the microscope after the end of the bleeding measurements. In some cases also microphotographs were taken (see fig. 11).

The results of the single experiments are computed in table 3. Squeezing alone retards the bleeding to a value, amounting to 67—80 % of that of the intact root. The retardation increased with increasing distance of the squeezed zone from the tip, a result in accord with the effect of cutting off shorter or longer parts of the apical end of the roots (cf. table 1). There is a marked difference between the experiments in table 1 and those in table 3. Whereas removal of 10—15 mm. of the apical part lowered the bleeding to 59 %, squeezing of the zone 10—17 mm. only reduced it to 82 %. Removal of 25—30 mm. left only 33 % of the bleeding whereas squeezing of a zone 20—27 mm. from the tip gave the value 76 %. From this comparison the conclusion can be drawn that in the squeezed roots a considerable translocation occurs from the apical part through the squeezed zone. This conclusion is supported by the fact that removing the part of the root below the squeezed zone considerably reduced the total bleeding (see table 3).

The squeezing, no doubt, disturbed the translocation through the cortex,

Table 3. *The influence of squeezing and cutting of the cortex on the bleeding.* The bleeding of the roots before the encroachment=100. Average of two series.A. *Only squeezing.*

Distance of the squeezed zone from the tip in mm.	Bleeding in 30 min.	Bleeding after removal of the piece below the squeezed zone
10—17	82.2	—
20—27	76.0	36.0
30—40	67.0	41.5

B. *Squeezing followed by cutting.*

Distance of the squeezed zone from the tip in mm.	Bleeding in 30 min.	Bleeding after removal of the piece below the squeezed zone
30—40	70.1	42.5
40—50	56.0	—

especially in a radial direction, because the cell rows were partly separated from one another. The cells were still living, however, and showed intensive protoplasmic streaming. As a consequence of the partial inhibition of the translocation in a radial direction the ascending stream in the stele was deprived of a part of the solutes coming from the squeezed zone of the cortex. This is probably the origin of the reduction of the bleeding.

It is to be expected that also the translocation in longitudinal direction through the cortical tissue is disturbed by the squeezing. The experiments with vital staining, however, showed such a translocation to be rather unimportant. The experiments with intersection of the cortex clearly demonstrate the insignificant rôle of the cortex in the ascending sap movement. Table 3 A and B shows practically identical bleeding values from roots with only squeezed or with intersected cortex. The intersection which comprises a complete rupture of at least $\frac{3}{4}$ of the cortex (see fig. 11 A) has no additional effect on the flux of bleeding.

Of the c. 70 % remaining bleeding of roots, the cortex of which has been squeezed or cut at a height of 30—40 mm. (see table 3 A and B), about 40 % are due to the translocation within the part above the squeezed zone (=translocation from the cortex radially through the passage cells of the endodermis and into the vessels). The rest, about 30 %, must emanate from the part below the wounded part of the cortex. These quantities are consequently transported in the stele, a fact showing that a straight cut in the cortex does not interfere with the transporting functions of the stele. If the

stele, however, has been injured in some way, e.g., violently separated from the cortex or sharply bent, the transporting power declines to values around or below 40 % of the total bleeding instead of 70 % (in two cases as low as 25 %).

The dominating function of the stele as a conduit for the ascending sap stream is directly demonstrated in experiments implying an intersection of the stele at the intact, but squeezed cortex. The difficult operation succeeded in a few cases and always resulted in a reduction of the bleeding to the same values as after cutting off the whole piece below the wound. These experiments give further evidence in favour of our conclusion that no longitudinal movement through the cortex participates in the transportation of inorganic solutes to the aerial parts of the plant.

e. The transportation of water and solutes through the open channels of the roots

Open channels of transportation are the vessels of the metaxylem (see fig. 9 C), and the intercellular channels of the cortex which probably, similar to the intercellularies of the grass leaf, may serve as a conduit for water in periods of evident water requirement from the side of the aerial parts.

A hydrostatic suction of known magnitude can easily be applied to a decapitated plant or an isolated root by joining the capillary tube with a gas burette, operating by means of mercury. As earlier mentioned (11, p. 51), even a small suction power considerably increases the outflow of sap or water from the upper end of a root. New experiments showed an increase of the flux 2—6 times that of the normal bleeding at a suction power of only 100—180 mm. Hg ($\frac{1}{6}$ — $\frac{1}{3}$ atmospheres). As a rule, air from the intercellularies of the cortex escapes together with the water, a fact pointing to the formation of an atmospheric underpressure also in the bulk of the tissues. This underpressure indicates the existence of a certain transport resistance in the living cells. If the root tip is cut off and consequently the vessels opened the underpressure is considerably lowered and the outflow of air less marked. It can, however, continue for 2—3 hours, even from a decapitated root, especially at low temperature. It should to be mentioned that the presence of air bladders in the vessels and intercellularies tremendously retards the passage of water. If the air is removed under the influence of an intensive suction (e.g., 400 mm. Hg) water flows much more rapidly through a decapitated root than through an intact one.

A pressure of 250 mm. Hg sucked the following quantities of water (in mm.³/1 min.) through:

1.	2.	3.
<i>Root intact</i>	<i>5 mm. of the tip removed</i>	<i>14 mm. removed</i>
1.3 (= c. 15 times the normal bleeding)	13.0	22.2

In an intact root the water must pass through the living cells of the meristem and the cortex before it enters the vessels. If 5 mm. of the tip are cut off the central vessel is just opened (see fig. 8 A). The difference between 1. and 2. (see above) represents the filtration resistance of the meristem and adjacent histogens. Most of the water in case 2. probably passes through the central vessel, the opening of which has an area of about 0.001 mm.² (see p. 121). The difference between cases 2. and 3. probably lies in a somewhat larger diameter of the central vessel at 14 mm. than at 5 mm.; also the small vessels are a little better developed at the higher level. The quantity water flowing through a capillary tube obeys the law of Poiseuille:

$$\text{Water transport} = \frac{\pi r^4 p}{8 \eta l}$$

Here is r = the radius of the tube, l = its length, p = the suction pressure, η = the coefficient of friction. According to this law only a widening of the tube by about 12 % is needed to increase the transporting capacity from 13 to 22. As an illustration to the direct relation between p and the transported quantity the following experiment may be mentioned. A root was decapitated at a height of 14 mm. and exposed to two different suction pressures, 105 and 181 mm. Hg. The relation between the quantities of transported water was 1 : 1.78; the relation $p_1 : p_2$ was 1 : 1.72.

The following experiments illustrate the low transport resistance raised from the side of the cortex. Basal pieces of roots (30—40 mm. of the tip zone cut away) were exposed to a suction pressure of 200 mm. Hg. The water ran through the open channels at least 30—40 times faster than the normal ascending sap stream. If now the lower end of the root segment were clamped (see below) and the vessels and other open channels consequently closed the speed of the water was only slightly (from 0 to 20 %) diminished. These experiments gave the interesting and perhaps surprising result that the cortex is extremely permeable to water. As mentioned above it is imperative, however, that the air bladders are removed from the intercellular channels. The obscure »ventile mechanism», referred to in an earlier paper (11, p. 52), is probably nothing but the sudden rise of the water transport after the air has gone. As long as air is still present a rise of the suction pressure has little influence, but the active sap stream is not influenced by the air. A considerably higher speed of water is needed to

remove the air. In the intact plant the air probably escapes to the aerial parts. It is difficult to ascertain from which parts of the root the air comes, but the comparatively large quantities make it probable that most of it is released from the cortex and that the »extra» rush of transported water develops in the intercellular channels, functioning as a reserve conduit for water in periods of extreme water requirements. I have not been able to find out if the central vessel ever contains air. The smaller vessels probably never do. In deciduous trees, however, the large vessels are frequently filled with air.

The effect of a suction pressure of $\frac{1}{3}$ atmosphere is much larger in the case of isolated roots than in the case of decapitated seedlings, viz. c. 15 times the normal bleeding against a maximum of only 6 times. Seedlings with 3—5 roots also behave quite differently after the removal of the root ends. This encroachment changes only slightly the quantity of sap outflow through the stalk and leaves. The conducting elements in these parts are probably the limiting factors here. In experiments with two seedlings, the one carrying 6 roots of 50—70 mm. length, the other 3 roots of 90 mm. length, the quantity of water sucked through at 250 mm. Hg only amounted to 0.2 mm.³ per root and minute, whereas the isolated roots transported 1.3 mm.³ in the same time. These figures show that owing to the large resistance in higher levels the capacity of the root as a water-conducting organ can be utilized only to a small extent by the aerial parts of the seedling. The resistance formed by the tissues of the root has no influence under these circumstances.

The experiments hitherto presented have shown that the main part of the ascending sap stream flows through the stele. The cortex has no appreciable qualities as a longitudinally conducting element unless for the momentary needs of the tissue itself. The »long-line transport» is exclusively confined to the stelar elements. On the other hand, it was shown that the cortex feeds the ascending stelar stream along the whole extension of a 80—100 mm. long root by transporting, in a radial direction, the salts absorbed from the medium. This preferably »polar» direction of the movement of salts is perhaps partly due to the oxidation-reduction gradient between cortex and stele and the effect of this gradient upon the activity of the anion respiration system (see p. 110).

The fact that a segment of a root (=a decapitated root) actually exudes sap from the upper end, although the vessels are opened at the bottom, is not contradictory, however, to the assumption of an exudation of the sap in the vessels and the non-polar movement of this sap in the direction of the minimum resistance, similar to the flow of water in a capillary tube.

Table 4. *Experiments with basal segments of 90—100 mm. long roots, from which a part of the tip zone was cut off.* The upper end of the remaining basal segment was attached to a bleeding tube. Clogging was performed by means of a pinchcock. Average of 3—4 consecutive cloggings and free-cuttings of the lower end. Bleeding of the intact root=100.

Length of the removed tip zone in mm.	Bleeding from the upper end of the basal segment	
	Lower end open	Lower end clogged
28	34.5	76.0
30	37.7	63.1
60	11.1	28.6

It was found namely that the root segment bleeds at both ends. If the sap is exuded laterally in the vessels along the whole extension of the root it must flow out both up and down, and the relation between the two fluxes must depend upon the dimensions of the openings and of the relative intensity of exudation in the different levels. The results quoted in tables 1 and 2 are thus in full agreement with the idea of the whole process just reported. The crux of the experiment, however, is the demonstration of a reversal of the outflux from a root segment after the clogging of one end.

In the first experiments along this line gelatin and vaseline were tried as clogging agents. Gelatin worked unsatisfactorily, probably owing to its high permeability to water. Vaseline proved to be more successful. A capillary tube was filled with vaseline and the lower end of a root segment (30 mm. of the tip zone had been removed) was introduced in it. After determination of the bleeding from the upper end the lower end was cut free again. In two cases the bleeding from the upper end decreased 21—23 % after opening the lower end. In a third case the difference amounted to 47 %. These experiments demonstrated that the sap flow from the lower end of a root segment can be reversed after clogging the vessels at that end and seeks an outlet through the opposite end of the segment, thus a non-polar movement in the direction of the minimum resistance. If living cells of the cortex were involved in a longitudinally flowing stream the vaseline would certainly not sustain the pressure.

A more efficient closure of the vessels was attained by pinching the root end by means of a pinchcock. By alternately pinching and cutting free one root end (each time only c. 2—3 mm. were cut off) the observations of the reversal of the flux could be repeated several times with the same root. It should to be emphasized that the temperature must be held very constant not only during the experiments but also some time before. The reason for this is to avoid disturbing air bladders in the bleeding stream. The results of a number of experiments are computed in table 4.

The agreement between the results quoted in table 4 with those in table 2

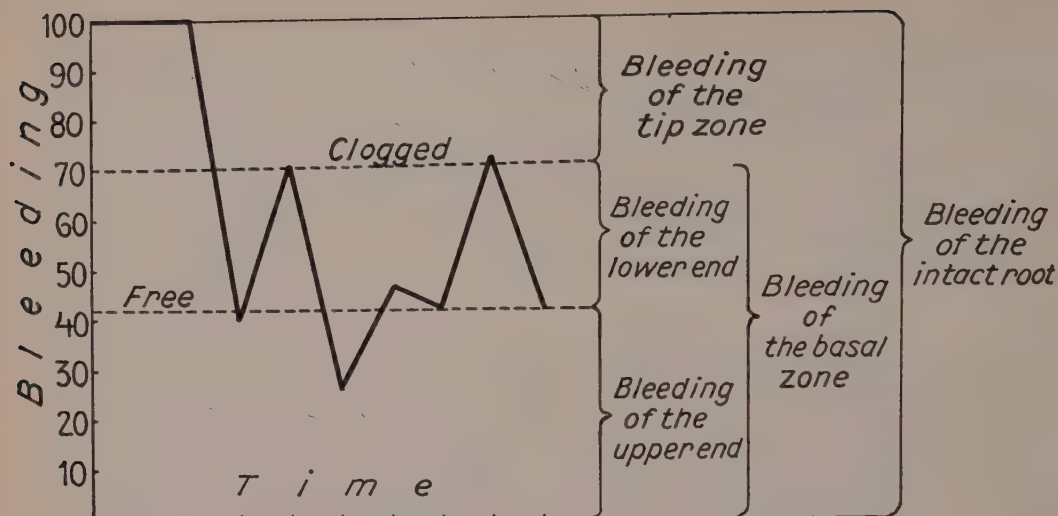


Fig. 12. Diagram of a bleeding experiment with alternating clamping and free cutting of the lower end of a basal piece of a root. Also the bleeding of the removed tip zone (30 mm.) was measured. All measurements are compared with the bleeding of the intact root before the experiment. Further explanation in the text.

is good. With 25—30 mm. of the tip zone removed the remaining root segment bled 42 % (table 2) compared with 35—38 % (table 4), or practically the same. The sum of the bleeding from both ends amounted to 70—73 % in table 2, to 63—76 % in table 4. Some results from one single experiment are plotted in fig. 12. Three successive cloggings and free-cuttings were carried out in this experiment and the first and third gave exactly the theoretically expected values. The deviating result of the second clogging is caused by incomplete closure.

Quite similar results to those in table 4 were obtained when the root segment was placed upside down and clamped at the anatomic upper end (see table 5).

The experiments with clogging of the openings of the vessels left no doubt as to the correctness of the thesis that what comes out from the lower end of an intermediate root segment is a fraction of the sap exuded in the xylem vessels. This fraction is regained if the outlets are clamped. An incomplete clogging, e.g., after cutting by scissors (see p. 118) correspondingly reduces the losses and increases the quantity of sap given off from the upper end. The fact that the losses from the opened vessels are comparatively smaller if the root is cut nearer to the tip probably depends upon the smaller dimensions of the younger vessels (see p. 121). As to the distribution of the

Table 5. *Experiments on reversed bleeding.* Original root length 90—100 mm. (secondary roots not yet appearing). Bleeding of the intact root=100. Constant temperature (see the text).

The tip zone (30 mm.)	Bleeding of:	
	The anatomically lower end of the basal segment	
	Upper end free	Upper end clamped
—	5.4	41.6
—	5.6	33.8
39.6	9.3	44.2

Table 6. *The distribution of the capacity of bleeding along the longitudinal axis of the root.* Determined by successive cutting and clamping of two roots of 85—100 mm. length. Relative values (total bleeding of the intact root=100).

Distance from the tip	1. (total length 100 mm.)	2. (total length 85 mm.)
0—20 mm.	22	38.3
20—40 »	32	26.9
40—60 »	20	20.3
60—80 »	15	14.5
80—100 »	11	—
	100	100

exuding power of the tissue in the centripetal direction along the axis of the root the experiments computed in table 6 show a certain dominance of the lower zones (20—40 mm.) but the individual variation is great, and even at 80—100 mm. a considerable power of internal exudation is still perceivable.

The fact was already emphasized (p. 131) that vaseline effectively stops the outflux at one end, if the opposite one is open; and that an exudation from living cortex would not behave in that way. The insignificant rôle of the cortex in the longitudinally flowing sap stream was, furthermore, demonstrated by some experiments with squeezing and cutting of the cortex near to the lower end of root segments.

In these experiments the root segments, after cutting off about 30 mm. of the tip zone, were inversely introduced into a capillary tube and the bleeding from the apical end determined. After that the root was squeezed and the cortex intersected a few millimeters below this end. After re-introducing the root into the capillary tube the bleeding was again determined.

A bleeding of 100 at the anatomically lower end corresponded to a mean bleeding of 74 (average of four experiments) after squeezing and cutting the cortex of this end. The reduction is comparatively small and of the same magnitude as the reduction of the bleeding after similar manipulations with

the cortex of intact roots (table 3). The explanation of the phenomenon, viz. a disturbance of the local radial exudation of the cortex, given on p. 127, is most probably valid also in this case.

Because in these experiments the zone of squeezing is situated so near to the end of the capillary tube a curious temporary effect probably of traumatic origin was occasionally observed, namely a sudden rise of the exudation immediately after the squeezing, followed by a slight retardation.

One main conclusion of the present investigation is the conception of a transference of the actively absorbed salts from the epidermis through the cortex and into the conducting elements of the stele. During this process the solutes preferably move in a radial direction, viz. perpendicular to the root axis. From the viewpoint of the ascending sap stream the longitudinal component of the movement of salts in the cortex has very little influence. On the other hand, it was concluded that the salts easily penetrate the cortex cells. These are provided with a comparatively high »salt permeability», a factor which is reported upon in the following section. It is questionable whether or not the movements in a longitudinal direction are directly conducted by the anion respiration. It seems more likely to assume that the transport of salts in the longitudinal direction is more of the non-polar passive type of rapid ion exchange (p. 111).

The fact that no exudation, or more accurately expressed no measurable exudation, was observed from the cross area of the cortex of a root segment does not necessarily mean that in the intact root no movement of salts occurs in this direction. Exposing the cellular interfaces to free contact with the medium may violently change the composition of the protoplasmic membranes and raise a barrier to a free ion exchange similar to that prevailing at the outer surface of the epidermis. It is difficult, however, to draw reliable conclusions from observations of changes in the distribution of salts in starving roots.

Such observations were submitted in earlier communications (11). Salts stored in the higher levels of the cortex disappear in periods of starvation (see fig. 3). This was attributed to a migration of the salts through the cortex in the direction of the growing tip. In the light of the present investigation it must be concluded that a considerable part of the salts present in the cortex of higher levels owing to the sap movement disappears in a radial direction. Earlier determinations of the active absorption of salts yielded an approximatively uniform uptake of 1.5—1.6 $\mu\text{mol}/\text{hour}/10\text{ mg. dry weight}$. Experiments on the salt exudation, presented below, gave values of quite similar magnitude (1.56 μmol) in respect to nitrate given off from

the basal parts of the roots (zones above 30 mm. from the tip). Because the exudation of salts to the ascending sap continued in distilled water (see 10) it must be concluded from these figures that the absorbed quantities of nitrate proceed directly into the stele. Only smaller quantities, if any, will then have the opportunity of moving down to the tip zone. In this zone only c. 50 % of the absorbed quantities of nitrate were regained in the bleeding sap (as an average of four experiments 0.685, against 1.5—1.6 μmol absorbed). This result tallies with the fact that 40—50 % of the absorbed nitrate is consumed in the nitrogen metabolism. The mentioned results show that the nitrate reduction is restricted to the tip zone where also the synthesis of new protoplasm has its site. The fact that the nitrate concentration in the growing tip under starvation soon declines to zero (see 11 and fig. 3), whereas the root continues to grow several days in distilled water, makes it probable, however, that some longitudinal salt transport really occurs, at least on short distances. There is also the possibility of a supply from the phloem of the stele. A downward flowing stream of salts must also operate in the first stages of the development of primary roots.

What is said here about a possible translocation in a longitudinal direction of the cortex is also valid for the living elements of the stele, especially the vascular epithelium, *viz.* the tissue provided with excellent qualities of exudation of salts. The major part of this exudation has now been shown to be the result of a non-polar process, its working component operating perpendicular to the interface between the epithelium and the vascular lumen. If in addition to this some polar flow in a longitudinal direction exists is very difficult to ascertain, but the experiments clearly show that a such possibility in any case must play a very subordinate rôle in the mechanism of sap movement. The same may be said about the endodermis. Its special functions are still obscure, but it was previously mentioned that its position as a boundary between the well aerated cortex and the tightly enclosed stelar tissue obviously creates a steep oxidation-reduction gradient possibly effective in pumping salts into the vascular system (on the diffusion of O_2 in onion roots see 2). Experiments with inhibitors of the anion respiration (cyanide, azide) on the other hand show that quantities of salts are emptied in the stele also without the aid of an accumulation mechanism.

No special problems are concerned with the interrelations between active sap movement and transpiration. The salts exuded into the vessels, together with certain quantities of water, are actively pressed through the whole vascular system of the plant. As the quantities of water thus pumped through the vascular system are rather limited, the importance of the transpiration obviously lies in its ability to drain off the salts from the place of exudation and to convey them to the zones of utilization in the aerial parts of the plant.

Table 7. *The exudation of nitrate from the ends of root segments.* Sap collected in cotton wool at 20° C. Number of the roots 17—36. The results are calculated per 1 root/hour.

Apical part of the root (tip zone; 32 mm.)	Basal part of the root (50 mm.)	
	Upper end	Lower end
0.00403 $\mu\text{mol NO}_3$	0.00796 $\mu\text{mol NO}_3$	0.00403 $\mu\text{mol NO}_3$

The cross section of the vessels has a total area of about 0.002 mm.². At a length of the active region of the root of 100 mm. the volume of the vascular pillars is 0.14 mm.³. A single root bleeds 0.05 mm.³/min. 20° C. The active region is consequently drained in about 3 minutes, if only the active ascending sap stream is working. The normal transpiration of a wheat seedling is about six times as large as the bleeding (10). It shortens the time of full drainage to 30 seconds. This value ought to be somewhat reduced according to the increasing resistance to the sap movement at the transition from root to shoot (see p. 130). The figures, nevertheless, illustrate the high efficiency of the root as the salt pump of the grass plant.

f. The exudation of nitrate and water in different zones of the root

The discussions in section A disclosed the fundamental fact that changes in the osmotical value of the cells caused either by exudation of salts or by metabolic disappearance of solutes create the motive power of the ascending sap stream, the hydrostatic pressure exerted arising to the turgor pressure of the exuding cells. It was previously shown (10) that the main constituents of the sap of wheat seedlings is potassium nitrate. It was also shown that the exudation of water and of salts occur simultaneously, but the relation salt : water is determined by (1) the osmotic value and the turgor tension of the exuding cells and (2) the quantity of »extra water» released according to the metabolic disappearance of osmotic material. These questions were thoroughly elucidated on the basis of an extensive experimental material, but no attempt was made to investigate the reaction of the different zones of the root. A number of new experiments were devoted to this problem.

One experiment with a bundle of 10 roots from which the bleeding sap was collected in filter paper yielded the value 0.1 $\mu\text{mol NO}_3$ exuded per root/hour at 24° C. In each one of two other experiments four intact roots were attached to capillary tubes and the sap collected after 30 minutes. The first series yielded 2.29 mm.³ sap per root hour, holding 0.10 $\mu\text{mol NO}_3$; concentration of the sap 0.044 mol/lit. The second series yielded 2.6 mm.³ sap/root hour, holding 0.09 $\mu\text{mol NO}_3$; concentration 0.035 mol/lit.

Table 8. *Exudation from the ends of basal root segments (67—75 mm.), after removal of 33—35 mm. of the tip zone. Both ends introduced in capillary tubes (fig. 5). Time of experiments 2 hours. Average of 4 roots.*

	Bleeding and nitrate exudation from:	
	Upper end	Lower end
Bleeding, volume	1.4 mm ³ /1h	1.59 mm ³ /1h
» nitrate.....	0.009 μ mol NO ₃	0.0077 μ mol NO ₃
Concentration	0.022 mol/lit.	0.015 mol/lit.

Table 9. *The relative exudation from the ends of a basal segment. Average from several experiments.*

Length of the removed tip zone in mm.	Exudation of nitrate from	
	The upper end	The lower end
20—25	100	57
25	100	63
25—30	100	98
35	100	127

The roots were taken from plants grown in nutrient solutions and consequently richly provided with nitrate. After 2 days in distilled water the concentration of the sap sank to 0.004—0.006 mol/lit., whereas the volume of the exudate amounted to 1.5—2.0 mm.³/root hour (3 parallels). These results are quite in accord with earlier results (10, 11).

Experiments computed in table 7 show about two times as much nitrate exuded from the upper end of the basal segment of the root as from the tip zone. The latter value is about the same as the quantity exuded from the lower end of the basal segment. The results agree fairly well with the measurements of the volume of the sap in table 2, thus conveying the idea that water and nitrate are exuded in approximately equal quantities from the different levels.

In table 8 also the volume of the sap was determined. In this case the sap coming from the lower end was a little more diluted than that appearing at the upper end. The same result was obtained in still another experiment in which 37 mm. of the tip were removed. As the sap collected from the lower end of a basal segment most probably is poured out from the cortex near this end the results point to the conclusion that a little more extra water is exuded from younger tissues than from older ones. This conclusion also tallies with the behaviour of the tip zone and is consistent with the general experience of the higher metabolic activity in the more apical parts of the root.

In the preceding pages the fact was repeatedly emphasized that on isolated basal root segments the bleeding from the lower end increases relatively to that from the upper end if the length of the removed tip zone is increased. In a number of experiments the simultaneous exudation of nitrate from both ends of basal segments was determined (see table 9).

The chemical determination of the salt exudation gives a further corroboration of the conclusions drawn in the previous sections of this paper. Under the ordinary conditions of the bleeding experiments the exudations of salts and water proceed uniformly along the axis of the root. The relation $\frac{\text{exuded salts}}{\text{exuded water}}$ slowly increases from the tip to the basal part of the root, *viz.* relatively more water is exuded in the younger parts.

g. The influence of inhibitors of the respiration and the glycolysis on the exudation of water and nitrate

Previous investigations (13) have dealt with the influence of a number of inhibitors on the absorption of anions and of the bleeding. It was shown that 0.001 m. potassium cyanide raised the volume of the sap to about 200 % during the first hour, whereas the absorption of anions from the medium was instantaneously inhibited. On the contrary, fluoride at all concentrations (0.001—0.01 m.) and all pH-values (4.8—7.1) acted as a specific inhibitor of the bleeding the effect rising with sinking pH, whereas the absorption from the medium was comparatively little influenced (0.01 m. NaF had no effect on the absorption at pH 7—10; at pH 4.5 the absorption was inhibited to 50 %). It was concluded that cyanide directly attacks the aerobic anion respiration, by screening off the active iron atoms of the enzyme system (see p. 108), but fluoride only indirectly over the glycolysis. It was also concluded that the bleeding, measured as exuded volume of liquid, is more directly related to the anaerobic glycolytic processes sensitive to NaF. Probably according to a Pasteur reaction cyanide indirectly, by blocking the end-oxidation, stimulates the glycolysis and concomittantly the bleeding, but the inhibited active transport of anions slowly chokes the exudation.

These previous experiments on bleeding were all performed with decapitated seedlings or intact roots and the observed effects of inhibitors thus comprehended the joint action of the different parts of the roots. A new experiment on the bleeding under anaerobical conditions is plotted in fig. 13. In a series of new experiments the bleeding of the isolated tip zone (25—35 mm.) was separately determined and also the exudation from the

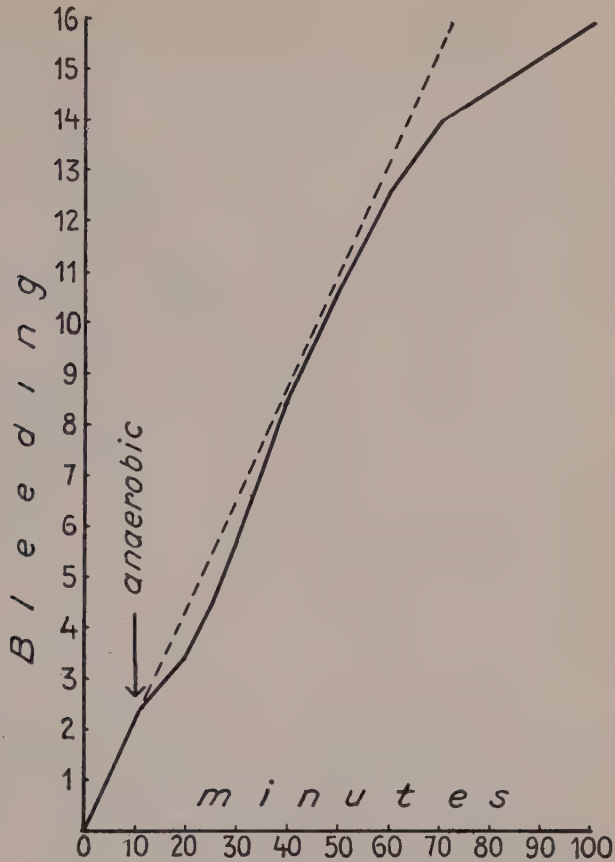


Fig. 13. The course of the bleeding under anaerobic conditions. The root was enclosed in a narrow tube with water free of oxygen. The dotted curve shows the course of the bleeding in air.

upper and lower ends of the remaining basal segment (about 50 mm.). The results of two significant experiments are plotted in figs. 14 and 15.

In 0.001 m. KCN (fig. 14) the bleeding of the isolated tip zone (in this case measuring 26 mm.) was decidedly stimulated. During the first hour of the treatment the increase was on an average 170 against 100 in distilled water. The stimulation continued during the second hour. The basal piece of the root, however, showed only a very slight or sometimes no stimulation of the bleeding of the lower end during the beginning of the first hour (average 100), followed by an inhibition (62 % at the beginning of the second hour). The bleeding of the upper end was rapidly inhibited (63 % during the first hour). These results show that the earlier observed stimulation of the total bleeding is exclusively caused by the reaction of the tip zone. Fig. 16 shows the similar reaction of a reversed basal segment.

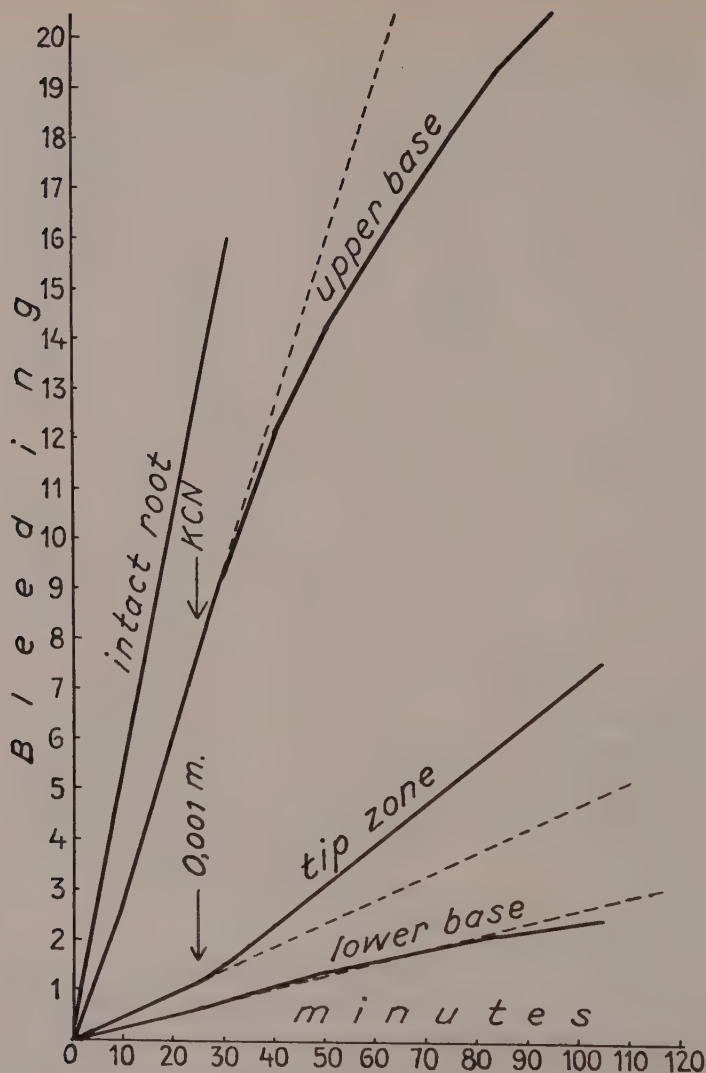


Fig. 14. Diagram of the course of the bleeding from an intact root, its tip zone, and its basal piece, after adding 0.001 m. KCN to the medium. The dotted lines show the bleeding of the tip zone, the lower and upper ends of the basal piece if the medium is only distilled water. Only the tip zone shows a stimulation of the bleeding (=the exudation of water) after treatment with cyanide.

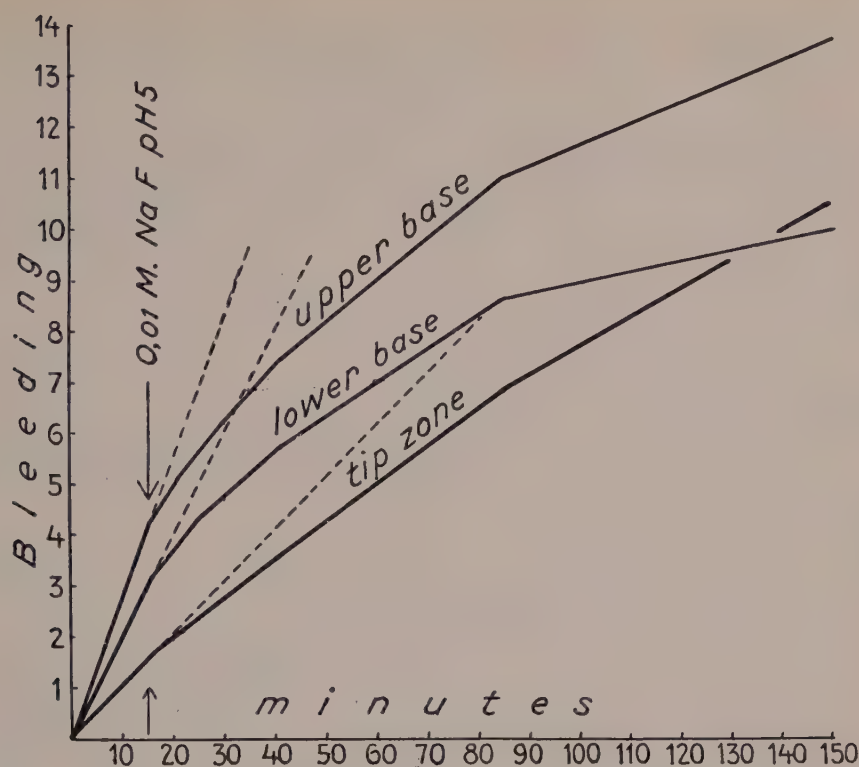


Fig. 15. As in fig. 14, but 0.01 m. NaF added instead of cyanide. Tip zone 26 mm., basal piece 60 mm. All zones show a strong retardation of the sap exudation.

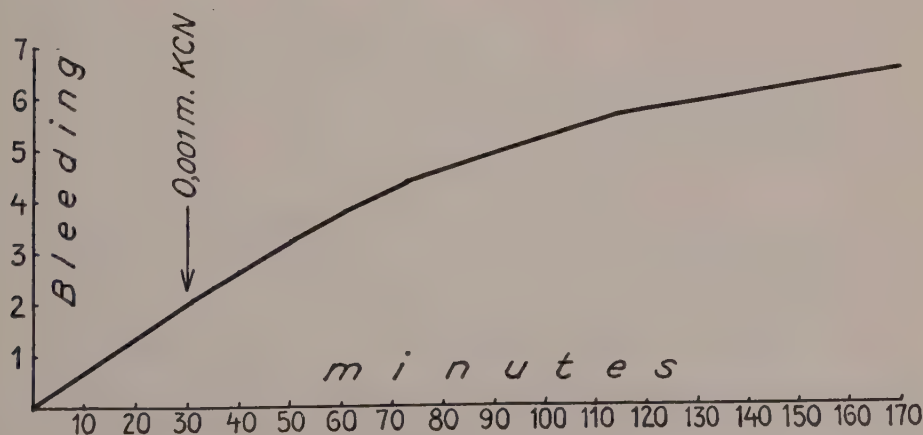


Fig. 16. The bleeding of a basal piece (68 mm.), after removal of a 15 mm. tip zone and the addition of 0.001 m. KCN. The root segment was reversed (anatomically lower end in the bleeding tube; cf. fig. 14).

Table 10. *The exudation of nitrate from the basal segment of wheat roots under the influence of cyanide and fluoride. Relative values; controls in aqua dest.=100. Removed tip zone 25—30 mm.*

A. *Medium 0.001 m. KCN.*

Time	Nitrate exudation from	
	The upper end	The lower end
1st hour	89	113
2nd »	66	55
3rd »	43	—

B. *Medium 0.002 m. KCN.*

Time	Nitrate exudation from	
	The upper end	The lower end
1st hour	30	31
2nd »	30	30

C. *Medium 0.01 m. NaF, pH 5.*

Time	Nitrate exudation from	
	The upper end	The lower end
1st hour	129	102
2nd »	77	32

The experiments with 0.01 m. NaF (+HCl, pH=5) also showed a difference in the reactions of the tip zone and the basal zones (fig. 15), but no stimulation appeared under the influence of this inhibitor. The bleeding of the isolated tip (length 32 mm.) decreased to 80—71 %, but the bleeding of the basal segment was more severely retarded, down to 39—17 %, practically identical at both ends.

The specific reaction of the tip zone is most probably caused by its characteristic metabolism. It was previously shown (14) that the tip zone (0—30 mm.), in addition to the ground respiration and the anion respiration which are also working in the higher zones has a third type of respiration characterized by its slow reaction to cyanide.

After this description of the exudation of water (volume bleeding sap) in different zones we now proceed to the exudation of nitrate. As a general result it was shown that exudation of water and of nitrate behave partly independently under the influence of inhibitors.

In the first series (table 10) a bundle of basal root segments was bent down in the solution in the shape of a U and the sap was collected by

Table 11. *Exudation of nitrate from the tip zone (30 mm.) and the basal segment of wheat roots. Bleeding from submersed objects. Relative values; controls in aqua dest.=100.*A. *Medium 0.001 m. KCN.*

Time	Nitrate exudation from	
	The tip zone	The basal segment
1st hour	85.5	91.5
2nd »	44.5	35.0

B. *Medium 0.002 m. KCN+0.001 m. HCl.*

Time	Nitrate exudation from	
	The tip zone	The basal segment
1st hour	52.5	50.0
2nd »	27.0	18.5

C. *Medium 0.01 m. NaF, pH 4.4.*

Time	Nitrate exudation from	
	The tip zone	The basal zone
1st hour	178	132
2nd »	160	100

means of cotton wool placed on the ends. A disadvantage of this method is the partly incomplete contact between the roots and the solution. The effects were also thoroughly weaker than in the arrangement tried below. According to table 10 the outflow of nitrate from both ends of the basal segments is very much the same.

A closer contact between the roots and the medium was secured in the experiments computed in table 11. Conductivity measurements showed (see 15) that no nitrates or other ionized compounds were exuded through the epidermis of roots treated with solutions of cyanide. All nitrate exuded from the submersed roots refers to bleeding through the ends.

As the anion respiration is completely checked in 0.001 m. KCN no re-absorption is to be feared. But in the case of the controls in distilled water re-absorption is always a possible source of error. As the roots were taken directly from nutrient solutions the ability of active absorption is low, however. Moreover, the concentration of the exuded quantities is very low in the dishes (after 1 hour about 5 $\mu\text{mol/lit.}$). For this reason an eventual re-absorption must proceed very slowly.

According to conductivity measurements (see 15) fluoride, on the other hand, induced a considerable exudation through the epidermis. The che-

Table 12. *Computation of some results from treatments of the roots of decapitated plants with 0.002—0.005 m. HCN (KCN+HCl) or O₂-free water. Experiments from 1943 (10).*

A. 0.002—0.005 m. HCN.

	0—2 hours	2—4 hours
Bleeding in ml/1g 1h	0.056	0.015
Exuded NO ₃ μmols	2.1	0.75
» K »	6.9	3.3
Conc. of NO ₃ millimols/lit. ...	4.2	7.1
» » K » » ...	15.8	28.4

B. Anaerobically.

	0—2 hours	2—4 hours
Bleeding in ml/1g 1h	0.060	0.028
Exuded NO ₃ μmols	1.3	0.87
» K »	3.1	1.6

mical composition of the exuded substances is not known but it is supposed that they are chiefly organic (nucleotides etc.). The quantities of nitrate exuded from submersed roots were altogether higher than the corresponding quantities exuded from the stele, but the differences are scarcely significant, because both cases showed higher values in the first hour, a phenomenon characteristic for the stelar exudation.

The series with KCN showed a slowly proceeding inhibition, the intensity of which attained about the same magnitude in the tips and in the basal segments. This result seems to indicate that the »third respiration» (see above) is not involved in the exudation of salts. The averages from 1—2 hours exudation in 0.001 m. KCN were 80.8 % and 63.0 % (separate collection of the sap and submersed root segments respectively). In 0.002 m. KCN+0.001 m. HCl the values are 40 % and 30 % respectively. This means that the inhibition rises steeply with the concentration of free HCN, but it, nevertheless, develops much slower than its corresponding action on the active absorption through the epidermis. The absorption of nitrate and chloride from the medium stopped immediately after adding 0.0001 m. KCN (see 11, tables 1 and 2). The active absorption begins in the surface layer hence the rapid response. On the other hand, the whole anion respiration of the root, amounting to at least 50 % of the total respiration, was inhibited by this low KCN concentration. This fact illustrates the rapid penetration of cyanide. As the exudation of nitrate continues for considerable time in all parts of the root it must be concluded that the process of internal exudation is not directly linked to the anion respiration.

Table 13. *Relative figures of the exudation of nitrate and water under the influence of cyanide and fluoride. Averages of observations covering a period of 2 hours.*A. *Cyanide series* (controls in aqua=100).

Part of the root	Exudation of nitrate	Exudation of water
Tip zone	65.0	170
Basal zone	63.0	60

B. *Fluoride series* (controls in aqua=100).

Part of the root	Exudation of nitrate	Exudation of water
Tip zone	169	78
Basal zone	116	28

This conclusion corroborates the results from 1943 (see table 12). Also in these experiments the exudation of nitrate slowly declined under the influence of cyanide, and measurable quantities were exuded even in the period 2—4 hours. Similar results were obtained anaerobically (table 12 B, cf. fig. 13). *The process of internal exudation thus proceeds independently of the aerobic respiration* (ground respiration, anion respiration and »third respiration«).

The experiments with fluoride, finally, illustrate the not forthcoming effect of the anaerobic glycolysis on the salt exudation (see table 10). This remarkable non-metabolic character of the internal exudation of salts is more like the outflow from a filled basin, slowly decreasing and finally ceasing if the basin is not refilled. The basin represents the stores of salts in the root tissue, originally provided by the anion respiration. The anion respiration is the up-hill active process in the whole cortical tissue (+epidermis). The salt exudation is the down-hill process. The centripetal direction of the salt flow is outlined by the barrier function of the epidermis together with the high »salt permeability« of the vascular epithelium.

As mentioned above the new experiments convey a modification of the picture of the co-operation between glycolysis and sap movement suggested in a previous paper (13). The glycolysis obviously does not affect the exudation of salts (nitrate), but it has a certain influence on the exudation of water (see table 13)

After treatment with fluoride the relation $\frac{\text{bl}}{\text{ex}} \left(\frac{\text{exuded water}}{\text{exuded salt}} \right)$ tremendously decreases, whereas the same, or even a larger, quantity of salts than under normal conditions is exuded. As a consequence of this the concentration of the bleeding sap, normally containing 30—40 millimols/litre, rises to about 120

millimols/litre. The exuded water is only $\frac{1}{3}$ to $\frac{1}{4}$ of the normal quantity. Of the sap from roots with normally proceeding glycolysis thus $\frac{2}{3}$ to $\frac{3}{4}$ must be regarded as »extra water» exuded according to the interaction of metabolism and osmosis. Cyanide, an inhibitor not affecting the glycolysis, does not change the relation $\frac{bl}{ex}$. In the basal root segments the exudation

of water and nitrate are both reduced to about 60 %. The large rise in the exudation of water in the tip zone (see table 13), however, is consistent with our concept of a stimulation of the glycolysis under the influence of cyanide (=Pasteur reaction). It has previously been shown that the roots lose approximately up to 50 % more glucose under anaerobical conditions, viz. at inactivation of the anion respiration (11, table 7, p. 43). The aerobic and anaerobic glycolytic processes are most intensively active in the tip zone.

It was previously observed (see table 12) that under conditions inhibiting the anion respiration comparatively more cations than anions are exuded in the sap. This was ascribed to a shortage of salt anions because of the inhibited active transport. It is known that the tip zone has a surplus of cations, primarily potassium (see fig. 3), and these will probably have a tendency to escape together with bicarbonate ions, especially at a reduction of the content of organic acids (malic acid) abundantly present in wheat roots. It was hypothetically supposed that potassium ions in some way participate in the glycolytic mechanism or in the phosphorylation (13). In any case cations are abundantly given off under anaerobic conditions (11, p. 18), also through the epidermis.

h. The temperature coefficients of the nitrate exudation

The experiments on the exudation of nitrate under conditions of partial or total inhibition of the aerobic respiration and the glycolysis have convincingly illustrated the pronounced non-metabolic character of the process. From this point of view it is of interest to know if also the temperature coefficient reveals something of a fundamental difference between active translocation and passive exudation of salts. These experiments were performed along similar lines as the ones in the foregoing section, viz. root segments were submersed in dishes filled with about 20 ml. water or the solution of an inhibitor (see table 14).

The results fully corroborate the general scheme presented above. In distilled water the sap exudation has a Q_{10} of 2.25—2.37 or what may be expected of a process involving biochemical reactions. Under the influence of the inhibitors Q_{10} falls to or below 1.50, values characteristic of non-metabolic processes e.g., diffusion or ion exchange. The values are a little higher for

Table 14. *Experiments on the exudation of nitrate from root segments submerged in dishes of water or solutions of inhibitors (KCN or NaF). Averages of seven series. Tip zone=30 mm.; Basal zone=40 mm.*

A. *Exudation of nitrate in $\mu\text{mol}/10\text{ mg. dry weight and hour.}$*

	12° C			24° C		
	aqua	0.001 m. KCN	0.01 m. NaF	aqua	0.001 m. KCN	0.01 m. NaF
Tip zone	0.38	0.44	0.82	0.86	0.61	1.23
Basal zone ...	0.48	0.56	0.87	1.15	0.77	1.21

B. *Temperature coefficient (Q_{10}).*

	aqua	0.001 m. KCN	0.01 m. NaF
Tip zone.....	2.25	1.39	1.50
Basal zone.....	2.37	1.37	1.39

NaF than for KCN indicating an influence of the anion respiration. The latter is only partially inhibited by fluoride.

The absolute quantities of nitrate exuded were at 12° C higher than in water, at 24° lower, if KCN was used, or still slightly higher, if NaF was used. An acceleration of the exudation means either an enhanced »permeability» or the elimination of some factor working in the opposite direction. The salt permeability, or more accurately the intercellular ion exchange (see p. 111), is probably rather sensitive to all factors disturbing the metabolic balance and it is by no means improbable that many inhibitors induce increasing permeability. The fact that at least fluoride strongly increases the exudation of organic substances through the epidermis merits attention in this respect. Also 2,4-dinitrophenol, an inhibitor of phosphorylation, strongly increases the reversed exudation and concomitantly also the exudation of stelar sap. But in addition to such less accurately studied possibilities a quite distinct process is known working opposite to exudation, namely the anion respiration. According to presented results this process is active in all cells of the cortex and its chief purpose is the filling of the salt stores of these cells, a process opposite to the »passive» exudation and always to some extent delaying the latter. At low temperatures the intensity of the anion respiration is insufficient to more effectively counteract the exudation. At higher temperatures its influence is the more increased as Q_{10} surmounts that of the exudation. Concomitantly it may be expected that the level of concentration, viz. the steady state at continued supply of salts, will be

greater at higher temperatures. Further experiments have to test the validity of this conclusion.

The fact that the exudation at 12° C of roots treated with cyanide exceeds the controls in water supports the assumption made above that the permeability is affected by the inhibitors. The tremendous rise in the exudation in NaF at 12° C points in the same direction.

E. Summary and Conclusions

After an exposition of the osmotic fundamentals of sap movement and the theory of anion respiration a number of experiments are presented for the purpose of elucidating the single processes co-operating in the active ascending sap stream of wheat roots.

If a single root is divided into two parts the sum of the exudations from the upper surface of the apical piece and the exudations from the upper and lower end of the basal segment is approximately equal to the total bleeding of the intact root. The total bleeding is the integral of the exudations at all levels of a 100 mm. long root. The zones below 40 mm. from the tip exude together more than 50 % of the total bleeding.

The sap is exuded by the vascular epithelium into the open vessels. The movement in the vessels has a non-polar character, viz. it can be reversed by clogging the vessels at the upper or lower end of a basal segment. The salts move through the cortex preferably perpendicular to the axis. No longitudinal salt transport through the cortex participates in the sap movement, but a limited translocation of salts up and down can be traced, especially in the tip zone. This translocation through the lengthened cortex cells is probably exclusively confined to the nutrition of the still growing parts of the cortex.

Salts and water are simultaneously exuded, but the relation $\frac{bl}{ex} \left(\frac{\text{exuded water}}{\text{exuded salt}} \right)$ is variable. Starving roots, in which the salt concentration of the vascular epithelium is low, give off a more diluted sap than well fed roots. This is a consequence of the osmotic fundamentals of sap exudation. A second source of variations of $\frac{bl}{ex}$ is the extra water exuded according to the osmotic effects of metabolic processes. As a consequence comparatively more extra water is exuded from the tip zone than from the basal parts of the root; nitrate assimilation and other synthetic processes localized in the tip zone contribute to a more voluminous sap.

Some of these metabolic processes are of the type of aerobic or anaerobic glycolysis, seemingly preferably localized in the tip zone. The stimulation

or retardation of such processes is the reason why an increased volume of sap is exuded from the lower parts of roots exposed to solutions of potassium cyanide and, concomitantly, why a very small sap volume is exuded after treatment with fluoride.

Contrary to the exudation of extra water the exudation of salts (nitrate) is not stimulated by cyanide but slowly declines to a fraction of that of the controls in distilled water. Fluoride stimulates the salt exudation and consequently produces a very low $\frac{bl}{ex}$. The salt exudation proceeds independently of any metabolic process and shows a lower Q_{10} than the anion respiration. A high »salt permeability» prevails in the tissues inside the epidermis, enabling a comparatively rapid flux of salts into the stele. The idea was advanced that this »permeability» is more of the kind of an ion exchange at the cellular interfaces, because only salts, not organic substances are exuded by the vascular epithelium.

Anion respiration and a comparatively high exchange permeability together build up the steady state of salts, viz. the concentration level of salts in the root tissues, the former representing the up-hill reaction, the latter the down-hill reaction. At fully active anion respiration salts are actively absorbed from the medium and accumulated in the cells; the outflux is consequently to a certain extent held back. At retarded anion respiration the outflow of salts continues unrestrictedly, thus sometimes, as under the influence of fluoride or low temperatures, exceeding the outflow at simultaneously working anion respiration.

The anatomical-physiological construction of the root turns this biochemical mechanism into a polar flow of inorganic sap from the medium into the conducting vessels of the stele. Of fundamental importance are the principally opposite properties of the outer boundary of the epidermis and the inner boundary of the vascular epithelium. Only the first named boundary has been more closely studied. According to its chemical composition and electrical charge it functions as a barrier against losses of salt anions even at checked anion respiration. No such barrier is obviously operating between the cortex cells, at the endodermis, or at the boundary between the vascular epithelium and the vessels. According to the passage cells the endodermis does not raise greater obstacles to the flow of salts from the cortex into the stele even in higher levels of the root (up to 100 mm. from the tip). The transpiration stream too apparently utilizes the passage cells, but also the intercellular channels of the cortex probably serve as reserve conduits during very high demands. Xylem vessels are developed closely behind the stretching zone (less than 5 mm. from the tip) and experiments with vital staining (neutral red) visualize the rapid translocation

of solutes from the cortex into these vessels. The living tissues of the meristem and the cortex only slightly retard the stream of water sucked through the root, if an underpressure is applied to the cut basal end or to the aerial parts. It was noticed that the latter, or the transition between roots and shoot, to some extent choke the transpiration stream, the full capacity of the roots not being utilized. The same is the case in respect of the ascending active sap stream.

The anatomic fabric of the root favours the development of an oxidation-reduction gradient in the direction of the radius (cf. 2, 9, 11, 12), a gradient according to the theory of anion respiration serving as a guide to the preferably centripetal forwarding of the salts absorbed through the epidermis. But also the structural polarity of the enzyme systems is to be considered here. It is to be expected that the oxygen tension in the stele, especially in the higher regions behind the deliberately lignified endodermis, is very low. Judging from the experiments with vital staining the endodermis seems to be endowed with a prominent capacity of accumulation. Whether it is provided with a similar valve or barrier function as the epidermis is open to further investigation.

In the main the experimental results corroborate previous work on sap movement performed in this laboratory. It has been shown once more that the salts are exuded in the open vessels, that the anion respiration is the ultimate motive power of the ascending stream, and that in the tissues inside the epidermis a steady state prevails between active accumulation and passive exudation. On two important points, however, the new results convey a modification or extension of previous conceptions. Firstly, it was shown that a translocation of salts in the longitudinal direction through the cortex has no practical importance for the ascending sap stream, because the salts at all levels are transported primarily in the direction of the radius straight into the stele. Secondly, it was shown that the exudation of salts is not linked to the anaerobic glycolysis, only the exudation of extra water frequently dominating the volume of the exuded sap. The possibility is still open, however, that the extra water and the extra carbon dioxide liberated owing to glycolytic processes are responsible for the preponderant exudation of metallic cations (potassium) in cases of checked anion respiration and that this exudation of cations may to some extent facilitate the liberation of anions. Under normal aerobic conditions such processes are very little influential, however:

References

1. Bennet-Clark, T. A. & Bexon, D.: Water relations of Plant Cells. — *New Phytologist* 45: 5. 1946.
2. Berris, L. J. & Norris, W. E.: Studies of onion root respiration. 1. Velocity of oxygen consumption in different segments of the root at different temperatures as a function of partial pressure of oxygen. — *Biochem. et Biophys. Acta* 3: 593. 1949.
3. Burström, H.: The colorimetric Determination of Nitrate in Plant Materials. — *Svensk Kem. Tidskr.* 54: 139. 1942.
4. — Studies on Growth and Metabolism of Roots. I. The Action of *n*-diamylacetic Acid on Root Elongation. — *Physiol. Plantarum* 2: 197. 1949.
5. Guillaumond, A. & Gautheret, R.-J.: Recherches sur la coloration vitale des cellules. — *Rev. Gén. de Bot.* 53: 25. 1946.
6. Lundegårdh, H.: Die Permeabilität der Wurzelspitzen von *Vicia Faba*. — *Kungl. Svenska Vetensk. Akad. Handl.* 47: 49. 1911.
7. — Die Nährstoffaufnahme der Pflanze. — *Jena* 1932.
8. — Ionenkonzentration und Ionenaustausch in der Grenzfläche Protoplasma; Lösung. — *Biochem. Zeitschr.* 298: 51. 1938.
9. — Investigations as to the Absorption and Accumulation of Inorganic Ions. — *Lantbrukshögsk. Annal. (Sweden)* 8: 233. 1940.
10. — Bleeding and Sap Movement. — *Ark. f. Bot. (Kungl. Vet. Akad. Sweden)* 31 A, Nr. 2. 1943.
11. — Absorption, Transport and Exudation of Inorganic Ions by the Roots. — *Ark. f. Bot. (Kungl. Vetensk. Akad. Sweden)* 32 A, Nr. 12. 1945.
12. — On the Mechanism of Active Movement of Water and Solutes through Plant Roots. — *Discuss. Faraday Soc. No. 3*: 139. 1948.
13. — Growth, Bleeding and Salt Absorption as influenced by Substances which interfere with Glycolysis and aerobic Respiration. — *Lantbrukshögsk. Annal. (Sweden)* 16: 339. 1949.
14. — Quantitative Relations between Respiration and Salt Absorption. — *Lantbrukshögsk. Annal. (Sweden)* 16: 372. 1949.
15. — The Time Course of the Ion Absorption of Wheat Roots and the Influence of the Concentration. — *Physiol. Plant.* 2: 388. 1949.
16. Myrbäck, K. & Vasseur, E.: Ueber die Lactosegärung und die Lokalisation der Enzyme in der Hefezelle. — *Hoppe-Seylers Zeitschr. f. physiol. Chemie* 277: 171. 1943.
17. Robertson, N. & Wilkins, Marjorie J.: Studies in the Metabolism of Plant Cells. VII. The quantitative Relation between Salt Accumulation and Salt Respiration. — *Australian Journ. Scient. Research. Ser. B. Biol. Sc.* 1: 17. 1948.
18. Sutter, Erika: Ueber die Wirkung des Kohlenoxydes auf Atmung und Ionenaufnahme von Weizenwurzeln. — *Experientia*. 1950 (in print).
19. Wanner, H.: Untersuchungen über die Temperaturabhängigkeit der Salzaufnahme durch Pflanzenwurzeln. I. Die relative Grösse der Temperaturkoeffizienten (Q_{10}) von Kationen- und Anionenaufnahme. — *Ber. Schweiz. Bot. Gesellsch.* 58: 123. 1948.
20. Vardar, Y.: Untersuchungen über die Wasserbewegungen in untergetauchten Pflanzen. — *Istanbul Universitesi Fen Fakultesi Mecmausi, Sr. B.* 15: 1. 1950.

The Significance of Concentration for the Rate of Ion Absorption by Higher Plants in Water Culture

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I. Introduction

Relatively few authors have been concerned with the significance of the concentration of nutrient solutions for the rate of ion absorption by plants in water culture. Among the numerous papers by Hoagland and co-workers on ion absorption from 1920 to 1930, there is only one (Hoagland, Davis and Hibbard, 1928) in which this question is discussed. The authors cultured *Nitella* in solutions with different concentrations of bromide and they found that *Nitella* accumulates increasing amounts of bromide with increasing concentration of bromide in the solution. Wrangell (1928), Schmid (1930), Peipers (1930), and Kunde (1931) investigated the absorption by different agricultural plants of nitrate, ammonium, phosphate, and potassium ions by means of water culture experiments, the plants being cultured in solutions containing different concentrations of the ions concerned. As a rule the experimental solutions contained only one or two salts. They found that the rate of absorption of the plants for each of the four ions rose with increasing concentration of the ion in the nutrient solution.

In opposition to this, Kostytschew, in his text-book of plant physiology (1931, p. 114), maintains that plants absorb about the same amounts of salt from very dilute as well as from rather concentrated solutions. This statement of Kostytschew is cited by Gračanin (1932), who asserts that it is contradictory to the general view of the significance of concentration for salt absorption, hence he has investigated this problem further. He experi-

mented with young maize and barley plants, which were placed in solutions of primary calcium phosphate of different concentrations. The phosphate content of the solutions was determined after one and five days, and it was shown that the plants had absorbed increasing amounts of phosphate with increasing concentration of the solutions. The plants had absorbed almost fifty times as much phosphate from a solution containing 1 g P_2O_5 per l as from a solution containing 0.01 g per l. Gračanin concludes from his experiment that Kostytschew's statements are not correct. Furthermore it is his opinion that his own experiments indicate that the salt intake of the plants is subject to the general physico-chemical laws which are valid for the adsorption of ions on colloidal particles, a view which is also shared by Wrangell and her school. Furthermore Burström (1939, Table 23 p. 267) found that excised wheat roots absorbed increasing amounts of nitrate with increasing concentration of nitrate in the solutions.

Finally, Overstreet, Broyer, Isaacs, and Delwiche (1942) found that the potassium absorption of excised barley roots rises with increasing concentration of potassium in the solution. They used flowing culture solutions, to which radioactive potassium salt was added in increasing concentrations.

It appears from the papers quoted above that the general opinion is that the rate of ion absorption by plants rises with increasing concentration of the ions concerned in the nutrient solutions. Only Kostytschew's statements contradict this view and these appear to be refuted by Gračanin's experimental results, which have not been questioned later. The experimental results presented here all the same confirm Kostytschew's statements. The experimental results of the other authors quoted above are therefore incorrect and this is chiefly due to errors inherent in the experimental methods, as will be shown later in detail.

II. Methods

a. Water culture experiments. Rye and kitchen kale (*Brassica oleracea acephala fimbriata*) were used for the experiments. The plants were cultured in glass cylinders, 20 cm high, each containing 5 l of nutrient solution. The vessels were closed at the top by means of a galvanized iron cover with four holes. A plant is placed in each of these holes, and supported by a flat, perforated cork. In the centre of the cover is a smaller hole through which a glass tube for aerating the solution passes to the bottom of the vessel. The aeration is performed by means of a pump which sends a relatively strong stream of air through all the solutions for two minutes, followed by a pause of five minutes. The pump works automatically and is controlled by a special relay system. In a few experiments continuous aeration is used. The bubbling gives both a suitable oxygen tension in the solution, so that the respiration of the roots is not decreased, and also stirs the solution very vigorously, so that the difference in concentration caused by the ion absorption of

the roots is immediately equalized. The latter condition has been found to be of very great importance.

A solution containing four salts, consisting of three cations and three anions in the ratio shown in Table 1, is used as nutrient solution. This solution, prepared with glass distilled water, represents the highest concentration which is generally used (total salt concentration about 2.2 g per l). It is designated »conc. 1». Solutions of lower concentration are prepared by simple dilution of this. A solution, the concentration of which is $\frac{1}{10}$ of that given in Table 1, is designated »conc. 0.1» etc. The nutrient solution contains, in addition to the four salts given in Table 1 trace elements. The amounts of these per l are 0.4 mg $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ — 0.4 mg H_3BO_3 — 0.2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ — 0.1 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.05 mg $(\text{NH}_4)_2\text{MoO}_4$. During preliminary cultivation of the plants 5 ml of a 1 % solution of ferric citrate is furthermore added, and also every third day 0.5 ml of a freshly prepared solution of ferrous sulphate.

The plants germinated in moist gravel are transferred, when they have reached a suitable size, to water culture in solution »conc. 0.2». The plants are cultured in this for a month or more, during which period the solutions are renewed every time it appears that the plants have emptied the solution of nitrate. The pH of the solution is kept constant at 6.8—7.0. This long cultivation is necessary for the plants to reach a considerable size. For estimation of the rate of ion absorption, plants which have a rather large root system in proportion to the volume of nutrient solution are most suited. Since the number of ions absorbed per unit time is naturally greater the larger the plant is. The larger the root system, the larger is the absorbing surface, this has been shown for example, by Kreyzi (1932).

The experiments were carried out in a greenhouse in 1948 and 1949, partly during the months of April, May, and June, partly in September and October.

b. Methods of analysis. Potassium, calcium, and magnesium were estimated both in the solutions and in the plants according to previously described methods (Olsen 1942).

The nitrate content of the nutrient solutions is estimated colourimetrically by means of the phenol disulphonic acid method in the following way. To a suitable volume of the solution, possibly diluted, containing from 0.01 to 0.20 mg NO_3 , is added a drop of 0.1 N NaOH and then evaporated to dryness in a little glass dish on the water bath. 1 ml phenol disulphonic acid (30 g pure phenol + 200 ml pure concentrated NO_3 -free sulphuric acid heated on the water bath for six hours) is spread evenly over the dried residue in the dish. After standing for five minutes, 5 ml of water are added and then 5 ml 7 N NaOH, which produces a yellow colour. After making up to 20 ml with water in the measuring flask, the colour strength of the yellow solution is measured in the Pulfrich photometer, using filter S 43. The determination can be carried out very accurately. The method has previously been used for the estimation of nitrate in soil, but was not suitable for this purpose because both humus and chloride give rise to errors. Since the nutrient solutions used contain neither humus nor chloride, these sources of error are avoided.

The phosphate content of the nutrient solutions is estimated colourimetrically according to Denigé. To 20 ml of the possibly diluted solution, (0.005 to 0.04 mg PO_4 should be present in 20 ml) 2 ml of the molybdenum reagent (2.2 g ammonium molybdate + 32.3 ml conc. H_2SO_4 + water to 400 ml) are added and also three drops of stannous chloride solution. The latter is prepared freshly daily by heating 0.2 g

Table 1. *Composition of the nutrient solution (Conc. 1).*

Per liter	mg. per liter					
	K	Ca	Mg	NO ₃	PO ₄	SO ₄
0.843 g Ca(NO ₃) ₂ · 4H ₂ O ...		143		443		
0.507 g MgSO ₄ · 7H ₂ O			50			200
0.114 g KH ₂ PO ₄	33				80	
0.745 g KNO ₃	288			456		
2.209 g total salts.....	321	143	50	899	80	200

stannous chloride with 2 ml conc. HCl until complete solution and then making up with water to 20 ml. Five minutes after addition of the reducing agent, the colour strength of the solution is measured in the Pulfrich photometer, using filter S 72.

Total nitrogen is estimated in the dried plant material by means of a previously described modification of Kjeldahl's method (Olsen 1927). Total phosphorus is determined according to Denigé, after previous acid digestion of the dried plant material, and finally total sulphur is estimated by the method given by Bailey (1936).

III. Nitrate and phosphate absorption in rye

Four vigorous rye plants were placed in a complete nutrient solution of concentration 1.0 (see Table 1). Samples of 5 ml were then removed daily for nitrate determination for a period of about ten days after which the plants had removed all nitrate from the solution. The results of the experiments are shown in Fig. 1 in which the ordinate gives the nitrate concentration of the solution per l, the abscissa the times of sampling. It can be seen that the curves obtained is a completely straight line, indicating that the rate of nitrate absorption is independent of the concentration of the solution. In this experiment, the same amount of nitrate per hour (about 0.06 milligram equivalents [m.e.] per l) has been absorbed, whether the concentration of the solution has been 14 m.e. per l or 0.14 m.e. per l.

In order to determine the slope of the curve in the region of the lowest concentrations, the same four plants were transferred, at the end of the experiment, to a new, very much diluted nutrient solution, containing 0.025 m.e. NO₃ per l, with continuous aeration. Samples for nitrate determination were then removed at intervals of about four minutes. The result of this experiment is inserted in Fig. 1 in the top, right hand corner. It appears from this figure that the rate of NO₃ intake continues to be independent of the concentration of the solution, right down to a concentration of 0.003 m.e. per l. At concentrations below this value, the rate of absorption falls with decreasing concentration of the solution. The curve finally becomes

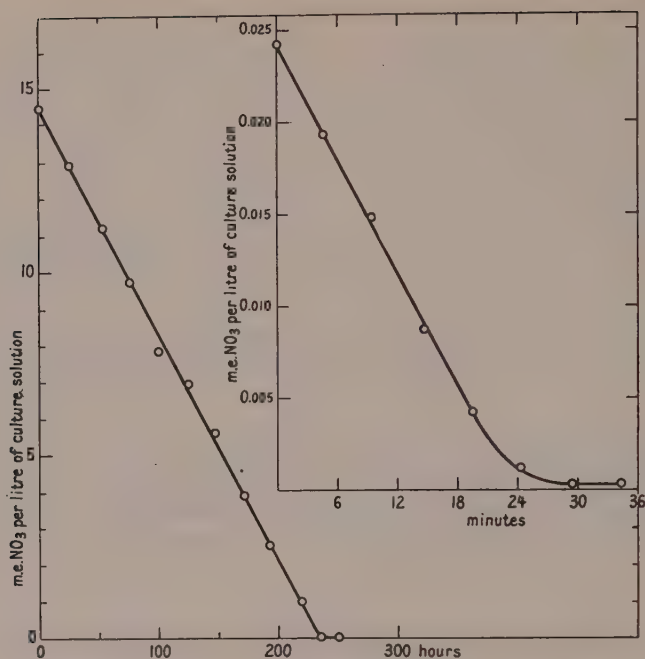


Fig. 1. *Nitrate absorption in rye.* The nitrate concentration of the nutrient solution at different times during the experimental period. The figure in the top right corner shows the lowest part of the curve to the left, enlarged 500 times.

parallel to the abscissa when the concentration of nitrate has reached about 0.0003 m.e. per l. and the plants do not seem able to reduce the concentration further.

Identical results are obtained with regard to phosphate absorption. Fig. 2 shows the result of an experiment on the absorption of phosphate in rye. The experiment is carried out in exactly the same way as the experiments described above on nitrate absorption, but with the difference that changes in the phosphate concentration of the nutrient solution are measured. In this figure also, the curve, which shows the concentration of the solution at different times, is a straight line from a PO_4 concentration of about 3.5 m.e. per l down to a concentration of 0.003 m.e. per l, after which it curves off. The lowest value to which the plant can decrease the concentration is also here about 0.0003 m.e. per l.

In addition to the two experiments on nitrate and phosphate absorption described above, many others were performed. In order to eliminate the effect of possible root growth during prolonged experiments, some of these were carried out as short term experiments by first determining the rate

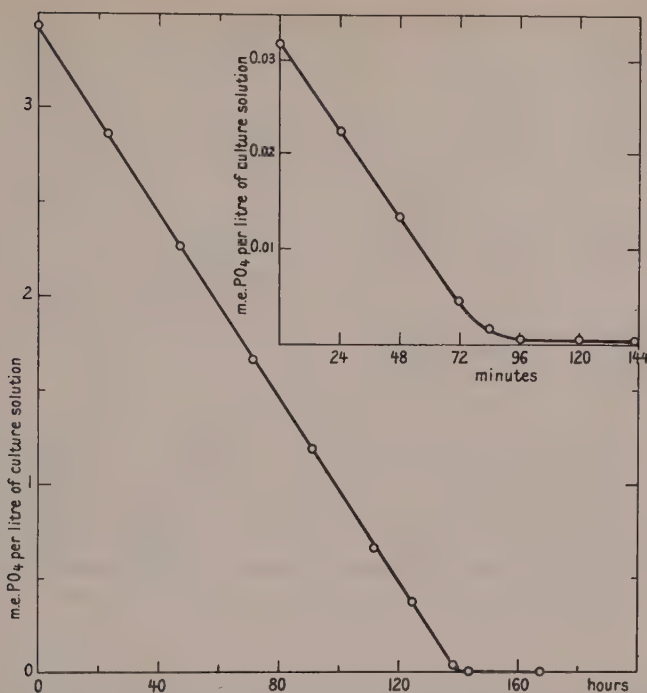


Fig. 2. *Phosphate absorption in rye.* The phosphate concentration of the nutrient solution at different times during the experimental period. The figure in the top right corner shows the lowest part of the curve to the left, enlarged 50 times.

of ion uptake in the plants from solutions of low concentrations, which only takes a short time, and then transferring the same plants to solutions with high concentrations. In this way it is possible to determine the nitrate and phosphate absorption of the same plants from solutions of two widely differing concentrations in a relatively short time. These experiments have all given the same result, i.e. that the rate of ion absorption of nitrate and phosphate is independent of the concentration of the nutrient solution, of course apart from the very lowest concentrations, which are below 0.003 m.e. per l.

There is the possibility that young plants, which were used in most of the experiments described in the literature, may behave differently. The following experiments were therefore performed. Eight day old seedlings of rye were placed in complete nutrient solutions having concentrations of 0.1, 0.3, and 1.0 (see Table 1). At suitable intervals, which gradually became shorter and shorter as the plants grew up, the solutions were renewed and the amount of nitrate absorbed by the plants was determined by analysis

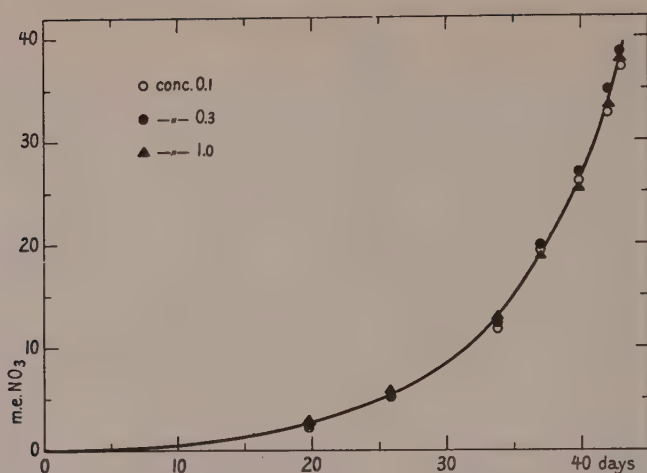


Fig. 3. Experiment with rye plants, which were 8 days old at the beginning of the experiment. The plants were cultured in solutions of 3 different concentrations, which were kept constant by frequently changing the solutions. The figure shows the amount of nitrate absorbed by the plants at different times.

of the solutions removed. The result of this experiment is given in Fig. 3, where by the use of special symbols for each of the three solutions, the amount of nitrate absorbed by the plants at different times, is given. The figure shows that the rate of absorption naturally increases very rapidly as the plant gradually grows, but the different concentrations of the solutions have practically no influence, the same amount of nitrate being absorbed whether the concentration of the solution is low or high. A similar curve was found for phosphate absorption.

IV. Total ion absorption in rye and kitchen kale

At the end of the experiments described above the plants were dried, weighed and analyzed for their contents of nitrogen, phosphorus, sulphur, potassium, calcium, and magnesium. The results of these analyses are given in Table 2, in which the amounts of nitrogen, phosphorus, and sulphur found are of course calculated as nitrate, phosphate, and sulphate respectively, in which form they are absorbed by the plants. The result of an identical experiment but with kitchen kale, is given to the right of the table. The values in Table 2 show clearly that the plants have, practically speaking, taken up the same amounts of the different ions, whether the concentration has been high or low. The cations potassium, calcium, and magne-

Table 2. Amount of different ions absorbed by rye and kitchen kale after 43 days in solutions of different concentrations. Figures are m.e. absorbed per 1 g dry weight.

	Rye			Kitchen kale		
	Conc. 0.1	Conc. 0.3	Conc. 1.0	Conc. 0.1	Conc. 0.3	Conc. 1.0
NO ₃	3.85	3.88	3.90	4.14	4.13	4.22
PO ₄	0.88	0.83	0.83	0.79	0.80	0.73
SO ₄	0.26	0.26	0.28	0.92	0.90	0.96
Total	4.99	4.97	5.01	5.85	5.83	5.91
K	1.60	1.53	1.58	1.75	1.72	1.78
Ca	0.25	0.26	0.28	1.51	1.45	1.46
Mg	0.27	0.29	0.29	0.56	0.55	0.57
Total	2.12	2.08	2.15	3.82	3.72	3.81
Dry weight g	9.9	10.4	9.7	14.1	15.0	13.8

sium are apparently absorbed independently of the concentration of the solutions, in the same way as nitrate and phosphate. Neither is the dry weight of the plants affected by differences in the concentration of the solution.

It is furthermore apparent from Table 2 that the sum of the anion equivalents absorbed is far greater than the sum of the cation equivalents absorbed, and that the nitrate equivalents absorbed alone are greater than the sum of the cation equivalents. The absorption of anions in excess of cations is, as is well known, due to the fact that nitrate absorption takes place chiefly by exchange of nitrate ions for bicarbonate ions, which are given off from the roots.

If the ion absorption of rye and kitchen kale are compared in Table 2, it will be seen that kitchen kale has absorbed nearly six times as much calcium as rye. Rye, like all the gramineae, absorbs very little calcium, kitchen kale, like most of the cruciferae, absorbs much calcium. There is also a characteristic difference with regard to the uptake of sulphate. Kitchen kale absorbs three and a half times as much sulphate as rye, the relatively high sulphate absorption of kitchen kale is characteristic for most cruciferae and is of course connected with the mustard oils present in these plants.

V. Discussion

The experiments described above show that the rate of ion absorption by higher plants in water culture is independent of the concentration in the solutions, of course apart from the very lowest concentrations lying below

0.003 m.e. per l. In the case of nitrate and phosphate, this is shown directly by following the changes in the concentration of the nutrient solutions, in the case of the other ions, indirectly by analyses of the plants. Direct experiments were also carried out for potassium and calcium, using the procedures described in III, and these experiments have given exactly the same results, i.e. that the rate of ion absorption is independent of the concentration of the nutrient solution.

These results may of course be rather surprising, since chemical and biological processes are generally dependent on the concentration, but it should be remembered that the ion absorption by plants is an active process, i.e., a process, for which energy is required, since it occurs against the concentration gradient, hence the results are in fact not so peculiar. If ion absorption were a passive process it would be dependent upon the concentration, there are however physico-chemical processes, which are independent of the concentration of the solutions, in which they occur, the electrolysis of a salt solution is a good example. In one respect, this process shows a certain similarity to the ion absorption of plants, since it also requires the transfer of energy. If a solution of copper sulphate is electrolyzed, the amount of copper deposited on the electrode per unit time is determined by the current strength alone, and that means the quantity of electricity, but the amount of copper deposited will be quite independent of the concentration of the copper sulphate solution.

As mentioned in the introduction, practically all previous investigators have come to the conclusion that the rate of ion absorption for a given ion increases with increasing concentration of the ion in the nutrient solution, this conclusion is in opposition to the experimental results described here. One of the reasons for this, and doubtless the most important, is that none of the authors mentioned have had any arrangement for stirring the solution. In the experiments described above, air has been pumped through the nutrient solution, causing a vigorous stirring. If the nutrient solutions are allowed to remain unstirred, the plants first absorb the ions from the parts of the solution which are nearest to the roots. Hence a considerable difference in concentration is caused in the solutions, which can only be evened out by diffusion, but, as is well known, this is a slow process. The greater the difference in concentration, the stronger, however, will be the stream of ions diffusing in, and therefore plants in stationary solutions are able to absorb greater amounts of ions from solutions with high concentration than from solutions with lower. This can be demonstrated experimentally. If aeration is stopped during an experiment, such as that the results of which are given in Fig. 1, it can always be shown that the rate of ion absorption decreases from the time aeration was stopped. The decrease was

very considerable, if aeration was stopped when the concentration of the solution was low, but the effect became less with increasing concentration, when aeration was stopped. Hence this shows that the effect of stirring is greatest at low concentrations and that, in the absence of stirring, relatively more ions are absorbed at higher than at lower concentrations.

One of the errors in Gračanin's experiment, described in the introduction, is the absence of stirring, but he has also committed two further errors. If the solutions of primary calcium phosphate, used by Gračanin, are prepared, it appears that his strongest solution has a pH of 3.1, his weakest a pH of 4.6. The rate of phosphate absorption, however, rises very rapidly with decreasing pH of solution, as will be shown in a later publication, and this is naturally a contributory cause to Gračanin's observation of increasing rate of absorption with increasing concentration of solution. Finally, Gračanin has presumably not used glass distilled water in his experiments, but ordinary distilled water, which generally contains about 0.2 mg Cu per l, and this amount of copper has a toxic effect on the plants and decreases the rate of absorption of the other ions, when the concentration of the solution is low, while copper does not have this effect, when the concentration of the solution is higher, owing to the antagonistic action of the other cations.

A more detailed description of the factors responsible for the previous investigators' findings of increasing ion absorption of plants with increasing concentration of the nutrient solution, will be forthcoming in a later paper.

In the experiments so far described, e.g., those, the results of which are given in Table 2, the concentration of the solutions as a whole has been varied, and that means that all four salts have always been present in the same proportion. The interaction of the ions has therefore been constant. If the concentration of the solution is not increased or decreased as a whole, but for example only the concentration of one of the four salts is altered, while that of the other three salts remains unchanged, a different result is obtained, the rate of ion absorption for the different ions being altered. The reason for this is that the mutual competition of the ions — the so-called ion antagonism — now shows its influence. If for example in a complete nutrient solution, the concentration of the potassium salt is increased, while the concentration of the other salts remains unchanged, the plants will absorb more potassium and it then appears as if an increasing amount of potassium is absorbed with increasing concentration of this ion in the nutrient solution. Simultaneously with the increased absorption of potassium, however, the absorption of calcium and magnesium decreases to such an extent that the total absorption of all cation equivalents per unit time remains unchanged (see Table 3). The rate of absorption of potassium is also in this instance no less independent of the concentration of potassium in the nutrient solution.

When more potassium is absorbed as the result of increasing concentration of the potassium salt in the nutrient solution, it is not due directly to the increased potassium concentration, but to the fact that the ratio of potassium to calcium and magnesium in the solution is now greater than previously. Exactly the same result, that is increased potassium intake, will be reached if the potassium concentration in the solution is not increased, but the concentrations of calcium and magnesium are decreased instead. Even if the concentration of potassium in the nutrient solution is decreased, increase in potassium intake, could be obtained, if only the concentration of calcium and magnesium are decreased still further than the potassium concentration. It is the proportion between the ions that is decisive, not the concentration of the individual ions as such.

The antagonistic effect is apparent in the experiments, the results of which are given in Table 3, but it also appears from previous investigations (Olsen, 1942), that if the proportion between the cations in a nutrient solution is varied, the proportion in which the plants absorb these ions is also varied, but the total absorption of cation equivalents is unaltered.¹

The same rules, which are valid for cations, can of course also be applied to anions.

The antagonistic effect between ions is thus not observed, when the total concentration of the solution is varied, as occurred in the experiments, the results of which are shown in Table 2, and since the solution in these experiments are continuously changed, the same proportion between the ions is maintained. If the solutions are not renewed, however, the plants as a result of their ion absorption, can gradually change the initial proportion, unless the solutions contain the ions in exactly the proportions in which they are absorbed, but this is certainly not always the case.

In the experiment, the results of which are given in Fig. 1, the solution had not been changed, the plants remaining in the same solution, until all nitrate was removed. In such an experiment, there is thus the possibility that the other anions can decrease the nitrate absorption, as the concentration of nitrate in the solution gradually diminishes. In this case it will be essentially a question of phosphate ions, but in the solution used nitrate and phosphate happened to be present in about the same proportion as that in which they are absorbed by rye, which means that the solution will be exhausted of nitrate and phosphate at about the same time. If the

¹ The fact that *Tussilago* in some of these experiments is an exception to this rule, is doubtless due to experimental errors, presumably to the fact that only the leaf blades were analyzed; this does not give a complete expression for the ion absorption of the whole plant, unless the ions are distributed evenly in the different organs of the plant, and this does not appear to be the case.

Table 3. *Experiment with kitchen kale.* Four plants were placed for 24 hours in a solution of conc. 0.2, then for 24 hours in a solution of the same composition, but in which the calcium content was increased five times, and finally for 24 hours in a solution of the same composition as the first, but with five times the content of potassium. The figures give the amounts of the three different cations absorbed in 24 hours, expressed in m.e. per litre.

Solution	K	Ca	Mg	K + Ca + Mg
Conc. 0.2	0.23	0.21	0.06	0.50
Ca \times 5	0.13	0.37	0.02	0.52
K \times 5	0.32	0.15	0.03	0.50

concentration of phosphate had been two or three times as high, the proportion of phosphate to nitrate would have been altered considerably as the content of nitrate gradually decreased, and finally the antagonistic effect of phosphate would have become evident as a decreased absorption of nitrate. In this case the curve would not have been a straight line, but would curve away from the abscissa. In order that the curve should remain rectilinear, it is necessary to have a continuously constant proportion between the anions.

Finally, it appears clearly that antagonistic effects can not play any part, when the plants absorb ions from a solution of a single salt, such as was the case, for example, in Gračanin's experiment. On the other hand, as mentioned in the introduction, Overstreet and co-workers' findings of increased potassium absorption with increased concentration of potassium in the nutrient solution is presumably due exclusively to the fact that only the concentration of the potassium salt is varied, while the concentrations of the remaining salts have been constant throughout the experiments.

Summary

The rate of ion absorption for a given ion is independent of the concentration of the ion in the nutrient solution, except for the very lowest concentrations, lying below 0.003 m.e. per l.

The number of ions absorbed per unit of time is constant, when absorption takes place from a single salt solution, or from a composite nutrient solution, in the latter case, however, only in as far as the proportion between the concentrations of the different ions in the nutrient solution does not change significantly during the experimental period.

If the proportion between the concentrations of the individual cations or anions in a nutrient solution is changed, the ratio in which these ions are absorbed by the plants is also changed.

The rate at which the individual cations or anions are absorbed from a composite nutrient solution is determined by the ratio between the concentrations of these ions in the nutrient solution, but not of their absolute concentration.

The total absorption per unit time of cation or anion equivalents is constant, that means that it is independent of the concentration of the solution and of the proportion between the ions.

References

- Bailey, K.: The sulphur distribution of proteins. — *Biochem. Journ.* 31:1396. 1936.
- Burström, H.: Über die Aufnahme und Assimilation von Nitrat durch Weizenkeimlinge. — *Ann. der landw. Hochschule Schwedens.* 7:247. 1939.
- Gračanin, M.: La concentration des ions comme facteur de la résorption. — *Compt. rend. Acad. Sci. Paris.* 195:1311. 1932.
- Hoagland, D. R., Davis, A. R., and Hibbard, P. L.: The influence of one ion on the accumulation of another by plant cells. — *Plant Physiology.* 3:473. 1928.
- Kostytschew, S.: *Lehrbuch der Pflanzenphysiologie.* Bd. 2. Berlin 1931.
- Kreyzi, R.: Beiträge zur Phosphorsäureaufnahme der Pflanze in Wasserkultur. — *Zeitschr. f. Pflanzenernährung Düng. u. Bodenk.* — A. 25:156. 1932.
- Kunde, W.: Ueber die Geschwindigkeit der Aufnahme von K-Ionen durch die Pflanze. — *Diss. Hohenheim.* 1931.
- Olsen, C.: On the determination of nitrogen in soils. — *Compt. rend. Lab. Carlsberg.* 17. Nr. 3. 1927.
- Water culture experiments with higher green plants in nutrient solutions having different concentrations of calcium. — *Compt. rend. Lab. Carlsberg. Sér. Chim.* 24:69. 1942.
- Overstreet, R., Broyer, T. C., Isaacs, T. L., and Delwiche, C. C.: Additional studies regarding the cation absorption mechanism of plants in soil. — *Amer. Journal of Botany.* 29:227. 1942.
- Peipers, F.: Über die Geschwindigkeit der Aufnahme von PO_4 -Ionen durch verschiedene landwirtschaftliche Kulturpflanzen. — *Diss. Hohenheim.* 1930.
- Schmid, K.: Ueber die Geschwindigkeit der N-Aufnahme landwirtschaftlicher Kulturpflanzen aus Ammon- und Nitratsalzen. — *Diss. Hohenheim.* 1930.
- Wrangell, M.: Ueber die Geschwindigkeit der Ionenaufnahme durch die Pflanze. — *Zeitschr. f. physikal. Chemie.* 139:351. 1928.

A New Method for Large-Scale Aseptic Cultivation of Higher Plants

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During work with some aspects of the nutritional physiology of wheat it was necessary to grow endospermless seedlings in darkness. As this meant adding carbohydrates to the nutrient solution, the cultures had to be germ-free, otherwise they would soon be overgrown by fungi and bacteria. At the same time it was necessary to obtain sufficient plant material for chemical analyses in an experimental period of 10—14 days. With this aim in view — the aseptic culture of a considerable number of seedlings — a rearing apparatus and a planting technique, which are comparatively easy to handle and fitted for use in the laboratory, have been worked out. Since the method has now been used in routine work for more than six months with excellent results as regards sterility, it may be justified to publish an account of the procedure.

Review of the literature

The earliest sterilizing and rearing methods have been reviewed by Grafe (11) and most extensively by Klein and Kisser (16). Already among these methods can be found the same principles for the construction of the rearing units as those used by later investigators; from the point of view of this work the culture chambers of Grafe and Smirnow are especially interesting (see Klein and Kisser [16], fig. 1 and fig. 16). Both these workers grew the plants completely isolated from the external atmosphere, and were able to obtain rather large-scale cultures, but due to the technical difficulties there was a high risk of infection.

The methods for sterile cultivation of higher plants can be divided in two main types. Many workers are content to keep the roots sterile while the shoots are allowed to grow freely in the air; in the other type of cultivation, the seedlings are kept in completely closed culture chambers. Using the first-mentioned procedure the plants may be grown to maturity without the hindrance of a small culture space, but, on the other hand, there is a greater risk of contamination than in a totally isolated rearing unit; furthermore it is possible to grow only one or a few plants in each vessel. The »open» method is clearly derived from older types — still used by Truffaut and Bezsonoff (28) — and has been modified in different ways by Bobko (4), Klein and Kissner (16), Weissflog (30) and Gerretsen (10). A similar culture technique was also developed by Virtanen, von Hausen and Karström (31), whose method has been used in extensive researches by themselves and others, for instance Barker and Broyer (2).

Aseptic cultures where the plants are completely isolated from the external atmosphere necessarily must be used with aquatic plants such as *Lemna* and *Spirodela*, as described, for example, by Clark and Roller (8), and recently by Thimann and Edmondsson (27). For terrestrial species a large-scale method was devised by McMillan (20), though he hardly succeeded in overcoming the technical difficulties. A more perfect culture chamber was invented by Hatch (13) — in fact this seems to be the most elaborate method hitherto described, though it requires some rather special equipment. For very small-sized plants Tanaka (26) devised several rearing vessels, both for water and agar cultures. Melin (21, 22) and later Björkman (3) grew sterile conifer seedlings in Erlenmeyer flasks for several years during their investigations on mycorrhiza; Åslander (34) described a similar arrangement, adding the nutrients through a Berkefeld filter. Single seedlings have also been successfully kept germ-free by Yamaguchi (33), Burlet (7) and Khan (15) — the two first-mentioned authors using endospermless corn in some of their experiments. — Embryo cultures have sometimes been continued to the seedling stage and further; details of such experiments are found for instance, in LaRue (18) and Tukey (29) — Borgström, too, using a similar method (5) — and in the review by Fiedler (9). The work of Kent-Ziebur et al. (14) on *Hordeum* is especially relevant in connection with this paper. Many authors, among others Burgeff (6) and Spoerl (25) have used orchids, and *Oenothera* embryos were cultured by Newcomb and Cleland (23), who had an agar medium in a cylindrical vessel covered by another vessel of slightly larger dimensions.

Though it is not a question of sterile cultivation, the interesting method of Spoehr (24) and Leopold (19) for culturing etiolated plants to maturity may

be mentioned here. — The claim of Woodford and Gregory (32), that their investigations on salt uptake have been carried out under aseptic conditions, seems hardly justified in view of their own description of the technique.

A striking feature among all these methods for aseptic cultivation of plants is that none of them — perhaps with the exception of that of Hatch (13) — are generally applicable. Most of the techniques described are intended for a special culture medium and can be used only for one or a few specimens. Furthermore almost no method of large-scale cultivation of terrestrial plants hitherto invented — i.e. cultures with more than 5 to 10 specimens in each rearing unit — has offered reasonable security against infection. The technique to be described here makes possible the safe cultivation on water cultures of 56 wheat seedlings per vessel without the use of any complicated equipment. It seems to offer good possibilities of modification in various directions, the chief limit of applicability being that the space does not allow unlimited breeding. If this obstacle is to be overcome the author should first consider some modification of Gustafsson's apparatus for the germ-free rearing of rats (12), though in this case a very complicated and expensive equipment is required.

The rearing unit

The rearing unit is schematically shown in fig. 1. It consists of the following parts:

1) A 1 liter beaker of Pyrex glass (*a*) containing the nutrient solution. A scratch is made in the glass, marking off a suitable volume of culture medium. Corresponding marks are engraved in the bent glass rod (*b*) allowing the cork holder (*c*; cf. fig. 3) with its loop of stainless steel (*d*) to be mounted before sterilizing, in such a position that its lower side dips in the fluid when the apparatus is put together. Four such cork holders can be placed in one beaker, giving a total of 56 plants in each rearing unit. — A detailed description of the planting technique is given below.

2) A glass jar (*f*) with a breach, 2 cm. wide and 1 cm. high, in the edge and a 0.5 cm. wide hole in the wall. The dimensions, 15×25 cm., have been decided upon as suitable for the thermostats of this laboratory.

3) A glass tube (*e*) with an inner diameter of about 3 mm., bent according to the figure. One end of the tube is filled with 10—12 cm. of closely packed cotton wool impregnated with cetyl-pyridinium chloride (CPC), a strong germicide of the anion active type (see for instance Kylin [17]). This impregnation is made with a 0.1 % solution, after which the cotton wool is dried in the air before being used.

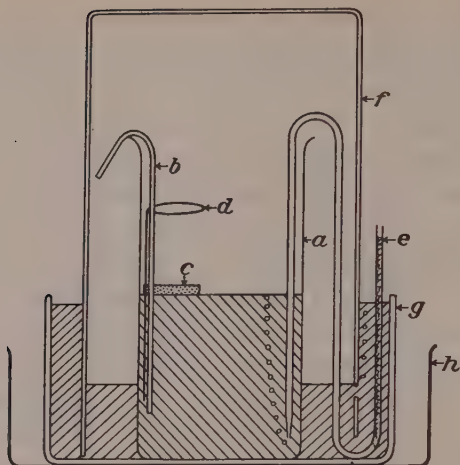


Fig. 1. *Schematic section of the rearing apparatus.* — The nutrient solution is kept in a Pyrex beaker (a). Hanging over the rim there are four bent glass rods (b) on each of which a cork holder (c) with a loop of stainless steel (d) is mounted. Air is let in through a bent glass tube (e) with a CPC-impregnated filter of cotton wool. The culture is covered by a glass jar (f) with an air outlet and a breach in the edge for the glass tube. The glass vessel (g) contains a saturated NaCl-CPC-solution constituting a bacterial trap and regulating the humidity. In case the liquid should flow over there is also an outer metal vessel (h).
— Scale 1 : 4.2.

4) A glass vessel (g), filled with a suitable amount of a concentrated solution of NaCl saturated with CPC (about 0.03 %). In case the liquid should flow over the brim, when the apparatus is in use, the vessel is placed in a metal container (h).

Before use the items of points 1—3 are carefully wrapped in tissue paper, and then, for mechanical protection, in wrapping-paper of the kind used in an autoclave. The parcels are sterilized in air of 150° C for two hours; if the heating is arranged so that this temperature is not reached until after about 45 minutes, even the large glass jar will stand the treatment.

Method of obtaining sterile surroundings

Of course a rearing unit of the type described is of no use unless a method is worked out for obtaining a sterile environment, where all operations can be safely carried out. This laboratory is furnished with a sterile room, equipped with a Westinghouse Sterilamp, and this provided the necessary basis for further progress. The following precautions have proved useful:

Before starting, the Sterilamp and a transportable Belmag mercury lamp are lit, and the working place washed with an alcoholic solution of 0.1 % CPC. A Bunsen jet is allowed to burn, and all necessary items are placed in the room and left for about 30 minutes. During this time a pair of coloured spectacles are prepared with an ointment protecting against the vapour formation, which will otherwise take place when wearing a mouth cover. The hands and arms are thoroughly washed, and a pair of rubber gloves put on and washed. When entering the transfer room the Belmag lamp is put out, whereas the Sterilamp is allowed to burn throughout the work. A white coat with short sleeves, always kept in the room so as to avoid undue contamination, is put on as well as an autoclave-sterilized mouth cover and a hood. To protect the eyes from the ultraviolet radiation it is also necessary to wear a sunshade and the coloured spectacles mentioned above. The gloves, arms, and working place are washed with the alcoholic CPC solution, and during the work this is repeated now and again.

In one operation, namely the sowing of sterilized seeds, it is better to omit the gloves. Autoclaving the gloves has proven unnecessary, and is of no significance if the work is carried out as intended by one man without assistants. — Tweezers and other tools are placed in a Petri dish and sterilized for two hours at 150° C before use; during the operations they are frequently re-sterilized by dipping in alcohol, which is then burnt off.

Sterilization and sowing of the seeds

Sterile wheat seeds are obtained by treatment with hydroxylamine according to a principle given by Bjälfve as used in this laboratory. The following modification has been used:

The kernels are pre-treated with mercury chloride and sterilized by placing them overnight in a 0.5 % solution of hydroxylamine hydrochloride. In the transfer room they are then washed three times with sterile water and sown in sterile Petri dishes, 19 cm. in diameter, containing a double layer of filter paper wetted by 40 ml. water. In view of the further handling of the seedlings it is of utmost importance that the kernels are placed with the ventral furrow downwards when sown; this is best carried out by using a pair of forceps with hooked points. After sowing, the Petri dishes are placed in a dark room of 20° C between sheets of sterilized paper, and in three days the seedlings are ready to be used. — Proceeding in the manner described will ensure maximal uniformity of the seedlings, and the number grown in one Petri dish will, as a rule, be sufficient for one rearing unit.

Planting and assembling the apparatus

The work in the sterile room is best performed according to the following prescriptions. The time needed to make one rearing unit ready is from 75 to 90 minutes.

1) The Pyrex beaker is taken out of its parcel, flamed, and charged with the appropriate nutrient solution, 500—600 ml. being a suitable amount. Since autoclaving a measured quantity of liquid will lead to losses through evaporation, the amount is made up to volume by pouring sterile water up to the scratch in the beaker (see the description of the apparatus above). Finally the beaker is placed in the vessel containing NaCl solution and covered by a disc of sterile, thick filter paper.

2) One of the Petri dishes with sterile seedlings is flamed and opened. The seedlings are freed from the endosperm and collected in a 14 cm. Petri dish containing sterile water. This operation is carried out according to fig. 2, using two pairs of forceps, one with straight and the other with hooked points. By pinching the straight one together the seedling is loosened from the endosperm, and can be totally freed and pushed into the collecting dish by applying the hook just behind it. When 60 seedlings are thus prepared, the planting begins.

3) One of the parcels containing mounted cork holders is opened, and the bent glass rod fixed in a big pair of forceps, held together by a Fisher »pinch cock» (fig. 3). Using an extra pair of preferably blunt-ended forceps there is no need of touching the glass rod and cork holder with the hands during this procedure. — In order to keep everything in a fixed position, the glass rod fits into a furrow which has been made in the forceps, and the legs of the pinch cock have been filed flat on the inside. The whole unit is finally flamed; if the cotton plug holding the cork disc in position is not too large, there is practically no risk of fire.

4) The unit of fig. 3 is held by the left hand, and the seedlings are mounted



Fig. 2. *Deseeding the plants.* — The seedling is loosened by pressing the straight pair of forceps (a) together around the endosperm. Using the hooked instrument (b) it is then completely detached and pushed into a collecting vessel.

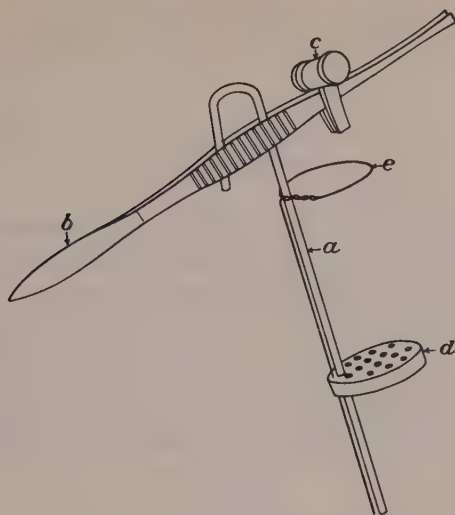


Fig. 3. *Planting unit*. — The glass rod (a) is mounted in a big pair of forceps (b) held together by a Fisher »pinch cock» (c). To keep everything in position the rod fits into a furrow in the pair of forceps and the pinch cock is filed flat on the inside. The coleoptiles are inserted from the under side of the cork holder (d), and the plants fastened by plugs of cotton wool. The shoots will be held erect by the loop of stainless steel (e).

on the cork holder. This is best done by a hooked pair of forceps, inserting *the coleoptiles from the under side of the disc*, which is held upwards. When three or four seedlings have been placed thus, the unit is turned right, and the plants adjusted and fixed in their positions by sterilized plugs of cotton wool. These are conveniently dipped in sterile water, since they are then much easier to handle. (As pointed out by Miss Berit Sandström, uniform plugs of cotton wool can be bought in appropriate sizes from a dentists' supply shop.) — When the whole cork holder has been planted, its position on the glass rod may be adjusted by means of a blunt-ended pair of forceps; it is then placed in the nutrient solution according to fig. 1.

5) When four cork holders have been planted and placed in the beaker, the parcel containing the bent glass tube is opened. The tube is seized by the end, which will remain outside the cylinder, flamed and placed in the beaker.

6) The glass cylinder is unwrapped and placed over the beaker according to fig. 1. The NaCl-CPC solution will then act as a liquid lock allowing no germs to enter inside the culture chamber; at the same time a constant humidity of about 78 % will be maintained during the experiment. The whole apparatus can now be moved to any place of suitable growing con-

ditions. Air under pressure is let in through the glass tube and will leave the cylinder through the hole in the wall, 5 to 10 litres an hour being a convenient speed.

Changing nutrient solutions

In my experiments I have changed the nutrient solution twice a week by transferring the seedlings into a new rearing unit. Following the instructions given below this work will require 20—30 minutes in the sterile room.

- 1) A Pyrex beaker is filled with fresh nutrient solution according to point 1) of the preceding paragraph.
- 2) The glass cylinder of the old culture chamber is removed, carefully so that drops from the germicidal solution do not splash on the seedlings. Before the work is continued the gloves should then be washed with the alcoholic CPC solution.
- 3) If the change is made for the first time the cork holders are again mounted as in fig. 3 with the aid of an extra pair of blunt-ended forceps. Using the same tool the loop of stainless steel is then adjusted at a suitable height, and the leaves of the seedlings collected within it, so that they will not adhere to the walls of the beaker, when placed in the new solution. — Later transfers can usually be made directly, using only the two pairs of pincers.
- 4) The new culture chamber is assembled as described above.

Remarks

The rearing unit should be placed in surroundings of constant temperature, or water will condense on the walls of the glass cylinder.

Employing the described methods in routine work, two infections have hitherto occurred in a total of about eighty plantings and transfers. One of these contaminations probably was due to faulty sterilization of the seeds, and the other seemingly was a one-spore infection of a *Penicillium*-resembling fungus when making a transfer. — As glucose has always been present in the nutrient solutions no special sterility tests have been deemed necessary, previous experiences indicating that a contamination is clearly visible in two days when the temperature is kept at 20° C. If required, samples for microbiological analyses can easily be taken in the transfer room by means of a sterile pipette.

No experimental results will be given in this paper. It will only be stated

that the roots of endospermless wheat seedlings give pictures closely resembling those found by Almestrand (1) in his cultures of excised roots of oats and barley.

Summary

A short review is given of the literature on sterile breeding of higher plants. A simple rearing apparatus is described. Instructions are given for its use with special regard as to the cultivation of endospermless wheat, 56 seedlings of which can be cultured in each unit.

The author is greatly indebted to Dr. Bengt Gustafsson and Artur Almestrand, Fil. mag., for their valuable advice regarding the technical difficulties involved in this work, and to Dr. Olof Kylin, who furnished him with a preparation of CPC.

References

1. Almestrand, A.: Studies on the Growth of Isolated Roots of Barley and Oats. — *Phys. Plant.* 2: 372. 1949.
2. Barker, H. A. & Broyer, T. C.: Notes on the Influence of Microorganisms on Growth of Squash Plants in Water Culture with Particular Reference to Manganese Nutrition. — *Soil Sci.* 53: 467. 1942.
3. Björkman, Erik: Ueber die Bedingungen der Mykorrhizabildung bei Kiefer und Fichte. — *Symb. Bot. Ups.*, VI, Nr 2. 1942.
4. Bobko, E.: Eine neue Methode der sterilen Kultur höherer Pflanzen. — *Ztschr. Pflanzenern. u. Düngung*, 3 A: 41. 1924.
5. Borgström, Georg: Anthogenesis in Etiolated Pea Seedlings. — *Bot. Notiser* 1939: 830.
6. Burgeff, H.: Samenkeimung der Orchideen. — Jena, 1934.
7. Burlet, E.: Ueber die pflanzliche Organkultur und ihre Anwendung bei physiologischen Untersuchungen. — *Ber. Schweiz. Bot. Ges.* 50: 519. 1940.
8. Clark, N. A. & Roller, E. M.: The Stimulation of *Lemna major* by Organic Matter under Sterile and Non-Sterile Conditions. — *Soil. Sci.* 31: 299. 1931.
9. Fiedler, H.: Die pflanzliche Gewebe- und Organkultur. — *Ztschr. Bot.* 33: 369. 1938.
10. Gerretsen, F. C.: Das Katadyn-Verfahren zur sterilen Kultur höherer Pflanzen. — *Planta* 23: 593. 1935.
11. Grafe, V.: Ernährungsphysiologisches Praktikum der höheren Pflanzen. — Berlin, 1914.
12. Gustafsson, B.: Germ-Free Rearing of Rats. — *Acta Pat. Microbiol. Scand. Suppl.* 73. 1948.
13. Hatch, A. B.: A Culture Chamber for the Study of Mycorrhizae. — *J. Arnold Arboretum* 15: 358. 1934.
14. Kent Ziebur, N., Brink, R. A., Graf, L. H., & Stahmann, M. A.: The Effect of Casein Hydrolysate on the Growth in Vitro of Immature *Hordeum* Embryos. — *Am. J. Bot.*, 37: 144. 1950.
15. Khan, I. U.: A Technique for Growing Citrus Seedlings under Aseptic Conditions of Culture. — *Phytopath.* 38: 756. 1948.
16. Klein, G. & Kisser, J.: Die sterile Kultur der höheren Pflanzen. — *Bot. Abhandlungen* 1, Nr 2. Jena, 1924.

17. Kylin, O.: Kvartära ammoniumföreningar som desinfektionsmedel. — Svensk Bryggeritidskr. 1949 nr 8.
18. LaRue, C. D.: The Growth of Plant Embryos in Culture. — Bull. Torrey Bot. Club 63: 365. 1936.
19. Leopold, A. C.: Flower Initiation in Total Darkness. — Pl. Phys. 24: 530. 1949.
20. McMillan, H. G.: A Method of Growing Bacteriologically Sterile Potato Plants. — U. S. Dept. Agr., Bull. 1465. 1927.
21. Melin, E.: Experimentelle Untersuchungen über die Konstitution und Oekologie der Mykorrhizen von *Pinus silvestris* und *Picea Abies*. — Mykol. Untersuch. u. Ber. von R. Falck 2: 73. Cassel, 1923.
22. — Untersuchungen über die Bedeutung der Baummykorrhiza. — Jena, 1925.
23. Newcomb, M. & Cleland, R. E.: Aseptic Cultivation of Excised Plant Embryos. — Science 104: 329. 1946.
24. Spoehr, H. A.: The Culture of Albino Maize. — Pl. Phys. 17: 397. 1942.
25. Spoerl, E.: Amino Acids as Sources of Nitrogen for Orchid Embryos. — Am. J. Bot. 35: 88. 1948.
26. Tanaka, I.: Studien über die Ernährung der höheren Pflanzen mit den organischen Verbindungen. — Jap. J. Bot. 5: 323. 1931.
27. Thimann, K. & Edmondson, Y.: The Biogenesis of the Anthocyanins I. — Arch. Biochem. 22: 33. 1949.
28. Truffaut, G. & Bezsonoff, N.: Fixation de l'Azote Gazeux par des Plantes Supérieures autres que les Legumineuses. — La Science du Sol 4: 1. 1925.
29. Tukey, H. B.: Growth Pattern of Plants Developed from Immature Embryos in Artificial Culture. — Bot. Gaz. 99: 630. 1938.
30. Weissflog, J.: Zur Sterilen Kultur der höheren Pflanzen. — Planta 19: 170. 1933.
31. Virtanen, A., von Hausen, S., & Karström, H.: Untersuchungen über die Leguminose-Bakterien und -Pflanzen XII. — Biochem. Ztschr. 258: 106. 1933.
32. Woodford, E. K. & Gregory, F. G.: Preliminary Results obtained with an Apparatus for the Study of Salt Uptake and Root Respiration of Whole Plants. — Ann. Bot. N. S. 12: 335. 1948.
33. Yamaguchi, S.: Studies on the Resorption of Urea by Root of *Zea mays*-Seedlings in Sterile Culture. — J. Fac. of Sci., Hokkaido Imp. Univ., Series V Botany 1: 37. 1930.
34. Åslander, A.: A Method for Growing Plants under Sterile Conditions. — Sv. Bot. Tidskr. 24: 111. 1930.

**Studies on Growth and Metabolism of Roots III.
The Adaptation against Growth Inhibition
by Aliphatic C₁₂ and C₁₁ Acids**

By

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In order to test whether the striking growth effects of di-n-amylacetic acid (DAA) (Burström 3, 4) are restricted to acids with a branched chain, experiments have been carried out with the two straight chain acids most resembling DAA, *viz.* undecanoic acid, $\text{CH}_3(\text{CH}_2)_9\text{COOH}$, and dodecanoic (lauric) acid, $\text{CH}_3(\text{CH}_2)_{10}\text{COOH}$. The preparations have been placed at our disposal by Dr. H. Veldstra, Amsterdamsche Chininefabriek, to whom we are very grateful. According to Veldstra and Booij (7) these acids should be physiologically less active than DAA, which also holds true with regard to their effect on roots. So far the experiments presented below have only led to a verification of results obtained earlier on other materials and by other methods. The observation justifying a full presentation of the data is, however, that marked differences occur in the sensitivity of roots of different age on one individual plant to the acids.

Such an apparent change in sensitivity or adaptation of a plant to a compound might be caused by the absorption of the compound or its destruction in the nutrient medium, especially if it were supplied in very low concentrations. In order to avoid such errors the experiments were carried out with flowing nutrient solutions.

The arrangement of the experiments was the same as used by Wilske and Burström (8); the test plant was spring wheat, and the experiments carried out at a constant temperature of 20° C. The cultures were started with just germinated seedlings, and run for six days illuminated by two 100 w. fluorescent lamps, under which the wheat seedlings grew well. The basic nutrient solution had the following

Table 1. *The growth of roots of control plants during six days.* — R_I denotes the main root, R_{II-III} the first pair of adventitious roots. Six experiments chosen at random. Increase in root length in mm.

Exp. No.	1	2	3	4	5	6
R_I	96	96	108	107	102	105
R_{II-III} ...	91	89	96	97	90	92
Average R_{I-III}	92	91	98	101	94	96

composition: $\text{Ca}(\text{NO}_3)_2$ 1/1000, KH_2PO_4 1/1000, MgSO_4 1/2000, FeCl_3 1/50000, and MnCl_2 1/50000 mol. The pH amounted to about 4.5 and increased to above 6 after the solutions had passed over the roots. This shows that a considerable absorption of nutrients took place, although each lot of 14 seedlings aged 2 to 8 days received about 750 ml. of nutrient solution daily. On the second, fourth, and sixth day 12 plants of each treatment were harvested, the roots measured and subjected to a microscopical investigation. — The technical part of these experiments has been carried out by Mrs. Greta Jansson.

In some tests the consumption of glucose and nitrate by sterile, isolated wheat roots was determined by means of routine methods of this laboratory (4).

At the time of transfer of the seedlings from Petri dishes, in which they had germinated, to the culture vessels three roots, the main root (R_I) and the first pair of adventitious roots (R_{II-III}), had developed. Their length amounted to 8 to 14 mm. During the six days of the experiment they increased in length by about 100 mm. Owing to the constant experimental conditions the growth of control plants, without additions of acids, was highly constant and only slightly but significantly lower for R_{II-III} than for R_I . Table 1 shows the growth of control roots in six consecutive experiments chosen at random. These three roots were joined together in the computations of the growth rates, and they reacted uniformly against the added acids. The table also shows the reproducibility of the tests; the controls were repeated in every series of experiments in spite of the good agreement. The second pair of adventitious roots, R_{IV-V} , was formed at the end of the experimental period, but on control plants these roots did not attain more than a few mm. in length before the readings were discontinued on the sixth day.

Dodecanoic acid

Figs. 1 and 2 show the result of one series of experiments with lauric acid supplied in the concentrations 10^{-6} , 10^{-5} , and 10^{-4} mol. In 10^{-6} mol

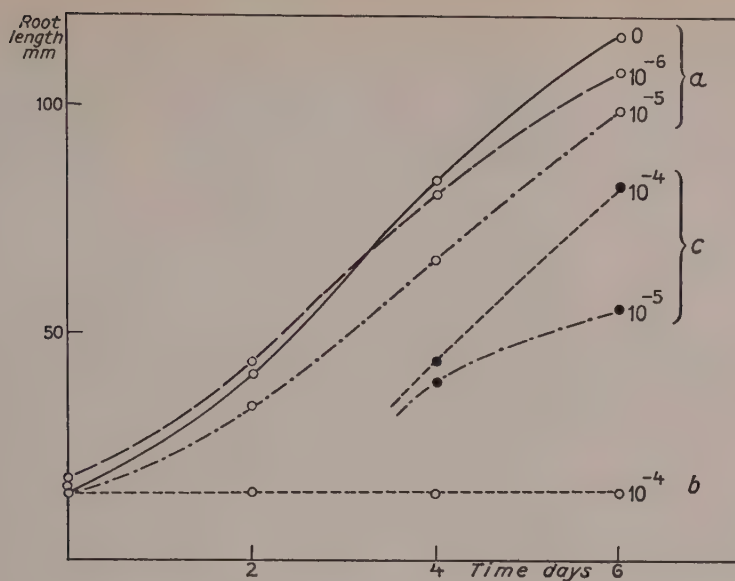


Fig. 1. The root elongation in solutions of lauric acid. Concentration of the acid given in mol. a denotes R_{I-III} , living; b R_{I-III} , dead in 10^{-4} mol; c R_{IV-V} .

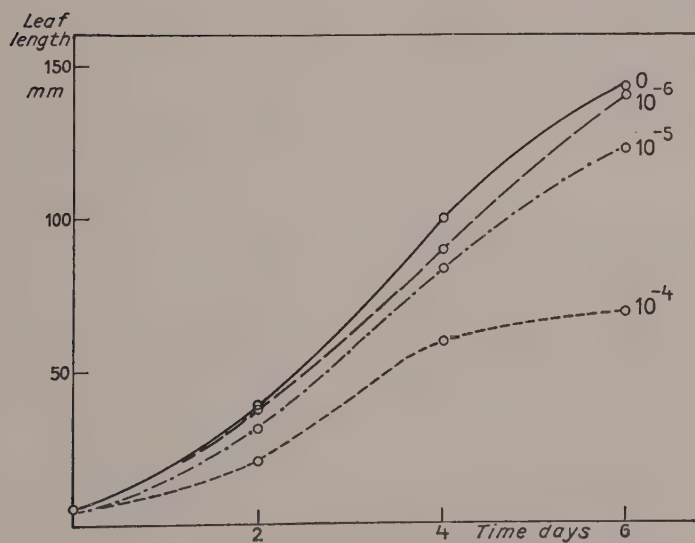


Fig. 2. The leaf elongation in solutions of lauric acid. Concentration of the acid given in mol.



Fig. 3. The appearance of plants grown for 4 days in lauric acid 10^{-4} mol. To the left control plant with R_{I-III} developed; to the right lauric acid plants, R_{I-III} dead, R_{IV-V} elongating.

there is no noticeable effect of the acid. In 10^{-5} there is an obvious delay in the elongation at the start; however, from the second day onwards the rate of elongation is nearly the same as in the control for R_{I-III} . Finally, in the highest concentration the initially present roots are instantaneously killed. Instead in 10^{-5} as well as in 10^{-4} mol the next pair of adventitious roots, R_{IV-V} , grow out and attain a length of about 40 mm. on the fourth day. The appearance of these plants and the controls are shown in Fig. 3, where the three dead initial roots are visible, as well as the rapidly and normally growing second pair. In 10^{-5} mol, which allows a normal rate of elongation of R_{I-III} this second pair lags behind, but in 10^{-4} mol R_{IV-V} elongate during the last interval as rapidly as R_{I-III} in the control. A microscopical study of the roots could not disclose any unusual structure or behaviour whatsoever of R_{IV-V} in the highest concentration. This behaviour of the roots was regularly observed also in cultures with stagnant solutions.

Two points should be emphasized. Firstly, the correlation between the successional pairs of adventitious roots; the second pair develops if the growth of the older roots is checked. This is nothing unusual, but a common phenomenon. Secondly, the remarkable intensitivity of R_{IV-V} to lauric acid. These roots develop from the start in

the strong concentration of the acid and are apparently wholly unaffected, although the initial roots are killed. If the plant is regarded as a unit it may be called an adaptation to the acid, but it is probably more correct to assume either that roots developing in the acid obtain other properties than those transferred to it, or that the successive pairs of adventitious roots *per se* have different properties. This question will be further discussed below.

To complete the picture of the plants in lauric acid attention should also be paid to the behaviour of the shoots. Fig. 2 shows the elongation of the first green leaf. There is a gradual retardation with increasing concentration of lauric acid, but the leaves remain healthy even in 10^{-4} mol. Whether this depends upon a low sensitivity of the leaves to the acid cannot be

decided without knowledge of the amounts of acid brought to the leaves. In any case, this circumstance should be especially noted, because in derivatives of the same molecular weight, which will be dealt with in another communication, the shoots are more susceptible than the roots.

Undecanoic acid

Because of the strong action of lauric acid it is difficult to study the effect of concentrations intermediate between the toxic and the inactive ones. In this respect tests with undecanoic acid are elucidating. The result of one series of experiments is shown in Fig. 4. Generally the action is the same as with lauric acid, although weaker.

The roots in 10^{-5} mol do not differ from the controls. The gross behaviour of the roots in 10^{-4} mol is the following. During the first two days there is little elongation of R_{I-III} , hence the second pair R_{IV-V} is formed. Between the second and the fourth day all five roots elongate, but then R_{I-III} are again checked and apparently die, whereas R_{IV-V} go on elongating at a

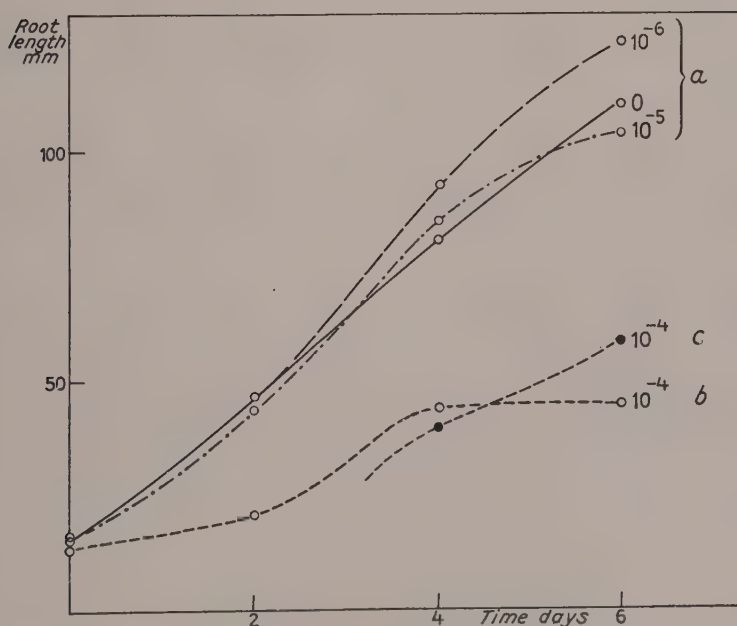


Fig. 4. The root elongation in solutions of undecanoic acid. Concentration of the acid given in mol. a denotes R_{I-III} living; b R_{I-III} dead; c R_{IV-V} .

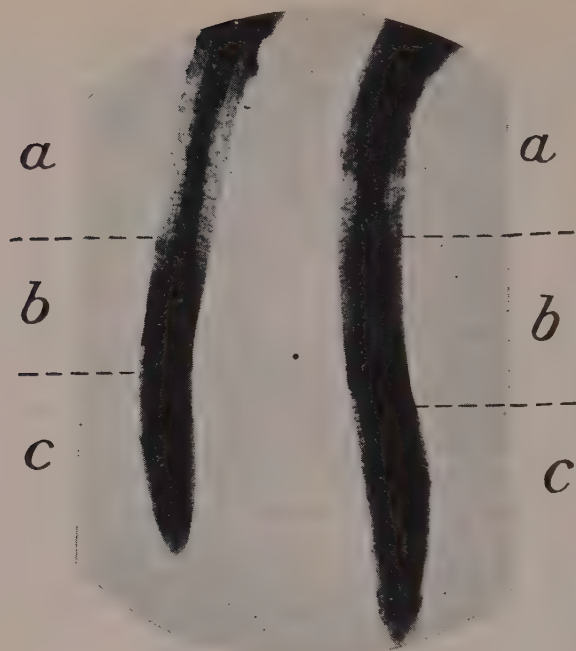


Fig. 5. *The appearance of roots in undecanoic acid 10^{-4} mol after two days. a — old mature part of the root; b — dying, brownish coloured zone of elongation; c — meristem with healthy, dividing cells.*

rate slightly less than that of the control roots. The same difference in susceptibility as with lauric acid is encountered here, and R_{IV-V} are under the microscope quite normally developed.

The picture of the roots after two days, illustrated in Fig. 5, gives a clue to the nature of this adaptation to the acids. Part *a* in the figure represents the old part of the root already matured at the transfer to the test solutions. Section *b* is brownish coloured and the cells are obviously dead; plasmolysis tests, which always are difficult to interpret with certainty, indicated that the cells actually were dead. This part represents the immature cells of the zone of elongation at the time of transfer; it is set off from the apical part by a sharp border line. The tip, part *c* consists of meristematic cells, apparently living and capable of elongation and also of division to some extent. Exact data of the rate of division within this part could not be obtained, however. The growth of this part is responsible for the elongation of the root during the second interval, but it is cut off from the basal part of the root by the dead zone *b*. Perhaps, owing to this lacking connexion

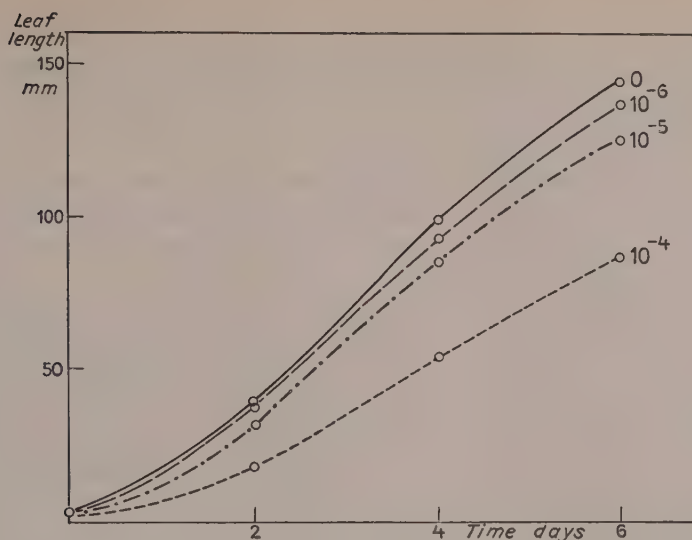


Fig. 6. The leaf elongation in solutions of undecanoic acid.
Concentration of the acid given in mol.

with the main part of the plant, the elongation is restricted, it ceases again after the fourth day, and the root finally dies. *It is obvious that only immature cells, actually in progress of elongation are affected by the acid.*

The cause of the statistically significant increase in root length with 10^{-6} mol undecanoic acid in Fig. 4 during the last interval is unknown. It has not been verified in other experiments, although a tendency in the same direction was found once at $3 \cdot 10^{-7}$ and 10^{-6} mol. Similar observations of slight growth acceleration after prolonged treatment with substances near the limit of inhibition were also made by Wilske and Burström (8). The deviations from the controls are too small in the present instance, however, to allow a detailed analysis by means of the course of the elongation curves.

The elongation of the leaves is inhibited by undecanoic acid, as shown in Fig. 6, to almost exactly the same degree as by lauric acid in corresponding concentrations, in spite of the differences in root development. This ought to be mentioned, but an interpretation must be postponed until experiments have been carried out with direct applications of the acids to the leaves.

Discussion

The specific inhibition of the cells of the initial zone of elongation was also observed with lauric acid. As a matter of fact, the initial delay of the

elongation of the roots in 10^{-5} mol lauric acid is of the same nature, but here the action is so much weaker, that the tip is able to overcome the difficulty with the dead basal section and can continue elongating normally. The only result of the treatment with the acid is the delay in the start of the elongation visible in Fig. 1. In such cases the affected zone is seen on the old root as a brown section near its base, and careful measurements of its location have confirmed its identity with the initial zone of elongation.

These results explain the behaviour of R_{IV-V} formed from meristems initiated in the presence of the acids but, of course, lacking elongating cells suddenly brought into contact with the acids. The high susceptibility to the acids is confined to cells in the course of elongation, formed in the absence of the acids, but does not occur with resting cells, nor in meristems or in derivatives of meristems formed in the presence of the acids. Seemingly this is a matter of an adaptation of the roots or the plants to the acids; this apparently implies that the meristems after being supplied with the acids change some properties so that the daughter cells become insensitive to the compounds. It may also be expressed in the following manner: that cells in progress of elongation are unable to stand such a change in the composition of the environment as an addition of these acids, whereas the presence of the acid in itself does not necessarily damage the cells. It is a question of definition as to whether this should be denoted as an adaptation or not.

Nevertheless, phenomena of this kind have been described earlier. Wheat roots can adapt themselves to auxin in a similar way (Burström 2). In this case already mature parts were sensitive to auxin and hypertrophied abnormally, whereas parts developing from meristems in auxin solutions behaved normally. The similarity to the actual case is obvious: parts formed in the presence of a compound are less sensitive than old ones. It may be assumed as a hypothesis, that undecanoic and dodecanoic acids cause some structural change within the cell. If this happens early in the meristem, it may be that the new structure is reproduced in the cytoplasmic growth, and a slightly modified but otherwise healthy cell results. If, on the other hand, it should happen later on during the elongation, the cell may have lost its power of rearranging its structure so that it fits in with the extraneous compound. In any case, the acids may be useful tools in studies of the structural changes during the elongation. Other cases of adaptation are described in the literature, for instance by Audus (1) and Gautheret (5), but whether they are of the same type cannot be decided.

Another point is also worth mentioning. When studying the comparative effects of acids of this group, it is obviously necessary to consider carefully the state of development of the cells and tissues treated with the acids, since

Table 2. *The assimilation of nitrate and consumption of glucose by tips of wheat roots in the presence of lauric and di-n-amyl acetic (DAA) acids. μ mol per 100 root tips; duration of exp. 48 hours; temp. 25° C. Initial fresh weight 0.48 g.*

Addition	fresh weight g.	nitrate μ mol	glucose μ mol
none	0.71	20.6	90
Lauric acid 10^{-5}	0.69	14.6	116
DAA 10^{-5} mol ...	0.81	0	106

different results can be obtained if tests are made on already growing cells or on meristematic parts.

This is obvious if these acids are compared with di-n-amylacetic acid. The important physical difference should according to Veldstra (6) consist in their different degree of lipophily. Physiologically they are similar in so far as they affect the root cells during their elongation, but otherwise their actions are very different. DAA continuously affects the elongation itself without any sign of an adaptation, whereas the straight chain acids have just the reverse action. Whether the actions of the three acids can be explained on a general basis by only a quantitative difference in activity seems rather unlikely at present, although the possibility cannot be wholly excluded. It seems, however, more probable in view of the histological pictures that they attack different points in the cell structure, perhaps according to their different lipophily. Anyway, the results emphasize the highly specific structure of compounds interfering in the different phases of the mechanism of cell elongation. The differential action on cells of different kinds is also a common feature of the two types of acids. It has not been possible, however, to demonstrate with lauric and undecanoic acid a different reaction of the external and internal layers of the root, although this does not imply that such do not exist.

Di-n-amylacetic acid has a striking inhibitory effect on the assimilation of nitrate, besides its influence on the cell elongation, apparently connected with the Mn and Fe interactions in this process. The Mn and Fe salts of di-n-amylacetic acid are very slightly soluble, which may contribute to this circumstance, but the corresponding salts of lauric and undecanoic acids should also be regarded as practically insoluble. Nevertheless, no action on the nitrate assimilation has been detected with lauric acid. Some figures given in Table 2 show no significant effect of lauric acid in 10^{-5} mol solution; in higher, lethal concentrations the whole metabolism is, of course, inhibited. Even on this point lauric and undecanoic acids markedly differ from di-n-amylacetic acid, and the effect of those acids is apparently more clearly involved only in the growth mechanism.

Summary

The action of undecanoic and dodecanoic (lauric) acid on the elongation of wheat roots has been studied in cultures with flowing nutrient solutions and under constant climatic conditions.

In medium concentrations, 10^{-5} mol lauric and 10^{-4} mol undecanoic acid, the cells in progress of elongation at the start of the experiments are killed, but meristems and mature cells remain unaffected. The former parts can go on growing and the roots thus overcome the poisoning.

If the initially present roots are killed, in 10^{-4} mol lauric acid, further adventitious roots develop in the acid-containing solutions without any disturbances of the elongation. It is concluded that cells in progress of elongation are specifically sensitive to these acids, but not meristems nor derivatives of meristems developed in the presence of the acids. The problem of adaptation to growth regulators is discussed from this point of view.

It is emphasized that the highly different sensitivity of cells of different stage of development must be taken into account in judging the growth effects of compounds of this type.

Undecanoic and dodecanoic acids do not share with di-n-amylacetic acid the property of interfering in the assimilation of nitrate, and the general physiological differences between the acids is emphasized.

References

1. Audus, L. J.: Studies on the phytostatic action of 2:4-dichlorophenoxyacetic acid and coumarin. — *New Phyt.* 47: 196. 1948.
2. Burström, H.: The influence of heteroauxin on cell growth and root development. — *Ann. Coll. Agr.* 10: 211. 1942.
3. — Studies on growth and metabolism of roots. I. The action of n-diamylacetic acid on root elongation. — *Physiol. Plant.* 2: 197. 1949.
4. — Studies on growth and metabolism of roots. II. N-diamylacetic acid and assimilation of nitrate. — *Physiol. Plant.* 2: 332. 1949.
5. Gautheret, R. J.: Sur les besoins en hétéro-auxine des cultures de tissus de quelques végétaux. — *C. R. Soc. Biol.* 141: 627. 1947.
6. Veldstra, H.: La balance hydrophile/lipophile des composés organiques, son importance pour leur action physiologique. — *Bull. Soc. Chim. Biol.* 31: 1. 1949.
7. — & Booij, H. L.: Researches on plant growth regulators XVII. Structure and activity. On the mechanism of the action III. — *Bioch. biophys. Acta* 3: 278. 1949.
8. Wilske, C. & Burström, H.: The growth-inhibiting action of thiophenoxy acetic acids. — *Physiol. Plant.* 3: 58. 1950.

Precursors of Tryptophan in the Nutrition of *Lentinus omphalodes* Fr. and some other Hymenomycetes

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Introduction

In an investigation of the nutritional requirements of a number of higher fungi (Fries 1950) it was observed that the Hymenomycete, *Lentinus omphalodes* Fr., responded very strongly to an addition of tryptophan. Since no fungus of such a physiological type had earlier been found, I considered it worth while to investigate more closely the effect on *Lentinus omphalodes* of tryptophan and some chemically related substances. These experiments, now to be described, had their natural starting-point in the recent findings, that tryptophan can be replaced by indole or anthranilic acid in the nutrition of certain bacteria, as well as in some tryptophan-less *Neurospora*-mutants. The unexpected discovery that aniline produced a strong, growth-promoting effect in *Lentinus omphalodes* led to a few experiments with some other, related fungi, in order to test their response to tryptophan and aniline.

Material and Methods

The strain of *Lentinus omphalodes* used in most of the experiments was isolated as a polysporous culture from a fruit-body found August 25, 1948, in a mixed wood at Valsätra c. 3 km south of Uppsala, Sweden. It was one of several fruit-bodies growing on small twigs of spruce and pine on the ground. The cultures of the other fungi studied in the two last experiments derived from various sources. *Lentinus lepideus* Fr., *Polyporus abietinus* (Dicks.) Fr., and *Polyporus annosus* Fr. were obtained from the stock culture collection of the Institute of Physiological Botany,

Uppsala. *Tricholoma fumosum* (Fr., non Pers.) was received by courtesy of Dr. Birgitta Norkrans; *Trametes heteromorpha* (Fr.) Bres., *Lenzites betulina* (L.) Fr., and *Peniophora fraxinea* (Pers.) Lll were isolated by me, the fruit-body of the last-mentioned species having been collected and determined by Dr. John Eriksson, Institute of Systematical Botany, Uppsala.

In all experiments the cultures were grown in 100 ml Erlenmeyer flasks of Jena glass. Each flask contained 25 ml of the ordinary synthetic medium, M 3, (glucose 20 g, ammonium tartrate 5 g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, NaCl 0.1 g, CaCl_2 0.1 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 4.43 mg, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 4.05 mg, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 4 mg, and distilled water 1 liter) supplemented with 40 μg thiamin per liter. This medium agrees completely, even as regards the quality of the ingredients, with that earlier described in experiments with *Ophiostoma* (Fries 1949), except that asparagine, inositol, and pyridoxin are omitted. The pH of the sterilized medium was 5.5. The compounds to be tested were usually sterilized separately in water solution by heating to boiling twice during two successive days, and then added to the culture flasks with sterile pipettes. The source of these chemicals is noted in the tables. The hydrolysed casein used in several of the experiments was always supplemented with tryptophan: 20 mg »NZ-case» (Sheffield Farms Co., New York) + 1 mg 1(—)-tryptophan (Eastman Kodak Co., Rochester, N. Y., USA) per flask.

After sterilization by autoclaving the flasks were inoculated with square pieces of mycelium, c. 2×2 mm, from malt-agar plate cultures. At least during the period immediately following inoculation, the mycelium developed entirely submerged hyphae. The cultures were incubated at $+25^\circ\text{C}$. At this temperature the daily increase in hyphal length of *Lentinus omphalodes* (on malt-agar) is 3.6 mm. The corresponding values for $+15^\circ$ and $+20^\circ$ are 1.9 mm and 2.7 mm, respectively.

When an experiment (or experimental series) was finished, the mycelium from each culture flask was washed thoroughly in distilled water, dried first at $+80^\circ$, then at $+100^\circ$, and finally weighed on a microbalance.

Each experimental series comprised six identically arranged cultures. The mean-square errors are not stated in the tables, except in Table 1, but they exceed 10 per cent of the average value only in some series, where the average weight is lower than 5 mg.

Experiments and Results

As was earlier found (Fries 1950), *Lentinus omphalodes* produced a good growth in the synthetic medium, M 3, if this was supplemented with hydrolysed casein to which a certain amount of 1(—)-tryptophan had been added. When a number of amino-acids were tested, it turned out that tryptophan alone could account for the main part of the growth-promoting effect of the amino-acid mixture.

This result was confirmed by repeated experiments. Table 1 shows that even the very small addition of 30 μg tryptophan per flask produced a significant effect. The optimal effect was reached by c. 500 μg tryptophan. Contrary to certain tryptophan-deficient mutants of *Neurospora* (Beadle, Mitchell, & Nyc 1947, Bonner 1948) *Lentinus omphalodes* cannot utilize nicotinic acid in place of tryptophan.

Table 1. *The effect of different amounts of tryptophan on the growth of Lentinus omphalodes. Incubation time 25 days.*

Substances added per flask	Dry weight of mycelium in mg	Final pH
Control	3.6 \pm 0.1	5.2
1(—)-tryptophan, 0.03 mg	5.8 \pm 0.2	5.0
» , 0.1 mg	7.9 \pm 0.3	5.0
» , 0.3 mg	11.1 \pm 0.6	4.9
» , 1 mg	14.4 \pm 0.5	4.8
Nicotinic-acid-amide, 0.1 mg ..	3.9 \pm 0.1	5.1
Hydrolysed casein, 21 mg	14.9 \pm 0.5	4.8

The effect of tryptophan was then compared with that of equimolar amounts of indole and anthranilic acid (o-amino-benzoic acid). As can be seen from Table 2 the three substances were almost equally active, except that indole in the highest concentration exerted an inhibitory effect which was not unexpected in view of the experiences of Cushing, Schwartz, & Bennett (1949) with this substance in experiments with *Neurospora*. The result shows that *Lentinus omphalodes* responds to these substances in the same way as do certain bacteria, in particular lactic acid bacteria (Snell 1943, Schweigert 1947), and some artificially produced mutations of *Neurospora* (Tatum, Bonner, & Beadle 1944). However, *Lentinus omphalodes* differs from these organisms in so far as it is actually capable of growing even without these particular additions, although with a much lower growth-rate.

In the next two experiments, the results of which are summarized in Table 3, the activity of a number of other chemically related compounds were tested. The most interesting result of these experiments seems to be the demonstration of a growth-promoting effect by aniline. With 1 mg of aniline almost twice as much mycelium was produced as in the control after 25 days. A slight activity of o-toluidine and isatin was also observed, although too small to be quite significant.

The response of *Lentinus omphalodes* to the three 3-indolecarboxylic acids was different in every case, if the higher concentrations, 100 μ m per flask, were considered (the lower concentrations were all inactive). 3-Indoleacetic acid was distinctly inhibiting, which could be expected from earlier experiences of various workers (Leonian & Lilly 1937, Murdia 1939, Bhargava 1946, Richards 1949). 3-Indolepropionic acid was quite neutral, while 3-indole-n-butyric acid evidently promoted growth.

The dipeptide, glycyl-l-tryptophan, was just as active as tryptophan alone. All other compounds tested were inactive, except the two purine-analogues, benzimidazole and 5-amino-7-hydroxy-triazolopyrimidine, which inhibited the growth more or less efficiently. o-Nitrotoluene was tested, since it can

Table 2. *The effect of different amounts of tryptophan, indole, and anthranilic acid on the growth of Lentinus omphalodes.*

Substances added per flask	Dry weight of mycelium in mg after		pH after 30 days
	30 days	40 days	
Controls	7.6	15.0	5.3
l(—)-tryptophan, 0.03 mg ..	11.4	18.8	5.1
» , 0.1 mg ...	14.0	19.5	4.9
» , 0.3 mg ...	18.5	23.0	4.8
» , 1 mg	30.0	38.0	4.5
» , 3 mg	27.5	45.2	4.6
Indole, 0.017 mg	10.0	17.7	4.9
» , 0.06 mg	11.3	20.9	4.9
» , 0.17 mg	16.0	26.8	4.8
» , 0.57 mg	30.8	36.4	4.5
» , 1.71 mg	5.4	13.3	5.0
Anthranilic acid, 0.02 mg ..	10.4	20.1	5.0
» , 0.07 mg ..	12.9	20.2	4.9
» , 0.20 mg ..	18.0	32.2	4.8
» , 0.67 mg ..	30.7	41.9	4.6
» , 2.01 mg ..	39.4	—	4.4
Casein hydrolysate, 21 mg ..	33.6	56.1	4.6
Yeast extract, 10 mg	30.9	34.8	4.7

be converted into anthranilic acid rather easily by treatment with hot alkali. The fungus, however, does not seem to be able to carry out this intramolecular rearrangement.

In order to corroborate the surprising result obtained with aniline, an experiment was performed, where the effect was examined of five different concentrations of aniline. It also included two series with kynurenic acid, a substance related to kynurenine, which in *Neurospora* as well as in *Drosophila* constitutes an intermediate in the formation of nicotinic acid from tryptophan.

The effect of aniline was confirmed (Table 4), 1.37 mg per 25 ml being the most active concentration (equimolar to 3 mg of tryptophan per 25 ml). It is difficult to compare exactly on a molar basis the activities of tryptophan, indole, anthranilic acid, and aniline, since the ratios change with time. After 30 days aniline was somewhat inferior, after 40 days superior to the other three substances as regards the growth-promoting effect. The optimal concentration seems, however, to be higher for aniline than for tryptophan. Kynurenic acid did not influence the mycelial growth in any direction.

The same experiment was repeated with the modification that ammonium tartrate was omitted in the nutrient solution. No growth occurred in any series, indicating that none of the substances tested could serve as a source of nitrogen to the fungus.

The importance of the time factor presented itself very clearly in the final

Table 3. *The effect of various substances on the growth of Lentinus omphalodes*. Incubation time 25 days. S.s.=saturated solution. B.D.H.=British Drug House; HLaR=Hoffmann-LaRoche, Basel; L & Co.=Light & Co., Wraybury, England; Merck=Merck, Darmstadt; S=obtained from Dr. E. Söderbäck, University of Uppsala, Sweden; W=obtained from Dr. D. W. Woolley, The Rockefeller Institute, New York, USA.

Substances added	Amount added per flask, µg	Dry weight of mycelium in mg	
		No tryptophan added	300 µg tryptophan per flask
Control	—	4.3	11.3
Indole (Coleman & Bell, Norwood, Ohio)	100	8.6	12.6
»	1000	4.0	3.1
Anthranilic acid (Kebo, Stockholm)	100	8.2	11.3
»	1000	15.0	11.2
Glycyl-L-tryptophan (HLaR)	30	5.5	11.8
»	100	7.2	13.3
»	300	11.6	12.1
»	1000	13.3	9.3
o-Nitrotoluene (Bofors, Sweden)	100	4.6	10.3
»	1000	5.0	10.7
o-Nitro-benzoic acid (B.D.H.)	100	4.6	10.5
»	1000	4.4	10.5
Carbazole (B.D.H.)	S.s.	5.5	9.7
Indigo (Merck)	S.s.	5.1	11.1
Isatin (Merck)	100	4.7	12.3
»	1000	5.8	12.9
Control	—	4.4	12.6
Anthranilic acid (Kebo, Stockholm)	100	9.0	12.8
»	1000	20.8	23.1
Aniline (Schering, Berlin)	100	5.9	14.3
»	1000	8.2	10.7
o-Toluidine (Merck)	100	5.5	11.6
»	1000	6.5	12.4
3-Indoleacetic acid (HLaR)	10	4.7	10.7
»	100	2.5	2.0
3-Indolepropionic acid (HLaR)	10	5.2	11.1
»	100	4.0	10.5
3-Indolebutyric acid (L & Co.)	10	5.0	11.6
»	100	8.1	13.4
Benzimidazole (W)	10	5.2	11.2
»	100	4.2	9.9
»	1000	4.4	7.6
5-Amino-7-hydroxy-triazolopyrimidine (S)	100	0.1	0.1
Pantothenic acid (HLaR)	1	4.2	10.7
»	10	3.8	11.8
Casein hydrolysate (»NZ-case»)	21000	20.1	18.9
Yeast extract (»Difco»)	10000	25.1	27.1

experiment with *Lentinus omphalodes* (Table 5). Obviously tryptophan produces the most immediate effect, which is quite distinct already after 15 days. After 30 days it was overtaken, and even surpassed, by indole and anthranilic acid, while aniline and 3-indolebutyric acid still were far behind.

Table 4. *The effect of different amounts of tryptophan, indole, anthranilic acid, aniline, and kynurenic acid on the growth of Lentinus omphalodes.*

Substances added per flask	Dry weight of mycelium in mg after	
	30 days	40 days
Control	5.6	14.6
l(—)-tryptophan, 100 μ g . . .	11.1	20.7
» , 1000 μ g . . .	19.1	32.1
Indole, 57 μ g	8.5	16.9
» , 570 μ g	19.3	33.0
» , 1900 μ g	0.4	0.6
Anthranilic acid, 67 μ g . . .	7.9	16.4
» , 670 μ g	21.2	31.3
Aniline, 45.6 μ g	7.8	18.5
» , 137 μ g	8.7	25.8
» , 456 μ g	13.1	27.7
» , 1370 μ g	16.1	40.2
» , 4560 μ g	13.2	36.1
Kynurenic acid, 92.7 μ g . . .	6.6	10.6
» , 927 μ g	5.9	11.2

After 50 days all these substances accounted for approximately the same relative increase in mycelial production. The growth then proceeded in all series, including the control, until all available glucose was consumed. This state was attained after c. 80 days.

Glucose-determinations made after 60 and 70 days for three of the series, viz. the control, and those with tryptophan and anthranilic acid, gave about the same value of the economic coefficient, 0.2.

Since next to nothing appears to be known about the effect of aniline upon the growth of fungi in general, it seemed justified to perform at least a preliminary experiment with some species related to *Lentinus omphalodes* in order to test their reaction to aniline and tryptophan. Like this fungus all of the seven species selected for this purpose were Hymenomycetes, and all, except one, were wood-inhabitants. This single exception was *Tricholoma fumosum*, a soil-inhabiting Agaric, which according to Dr. Birgitta Norkrans (personal communication) exhibits a weak positive response to tryptophan.

Tryptophan was tested in the concentration of 1 mg per flask, aniline in two concentrations, 456 μ g and 91.2 μ g per flask, respectively. (These latter quantities are equimolar to 1 mg and 0.2 mg, respectively, of tryptophan.) The time of incubation was adapted to the growth-rate of the species in question.

Table 6 shows the result of the experiment. The seven species behaved in the most different ways, but some interesting features may be stressed. Tryptophan either inhibited growth or was quite inactive, except in the case of *Tricholoma fumosum*, where a favourable effect was expected, and

Table 5. *The importance of the time-factor in determining the effect of tryptophan and some other chemicals on the growth of Lentinus omphalodes.*

μg of substance added per flask	Dry weight of mycelium in mg after days						
	15	30	40	50	60	70	80
Control	2.2	8.1	13.9	21.2	33.5	66.0	70.7
l(—)-tryptophan, 100 μg	3.6	12.5	21.1	25.5	43.3	62.7	83.0
» , 1000 μg	4.2	20.3	29.2	31.8	52.2	62.9	78.5
Indole, 57 μg	3.0	11.7	19.7	21.9	38.5	51.0	85.9
» , 570 μg	1.9	24.2	30.2	37.3	52.3	60.9	61.3
Anthranilic acid, 67 μg	2.9	13.2	18.7	25.8	38.0	54.8	87.7
» » , 670 μg	3.6	27.0	30.6	36.4	58.8	79.2	83.7
Aniline, 456 μg	2.3	13.0	19.7	32.2	41.8	59.3	87.3
3-Indolebutyric acid, 1074 μg	1.9	11.1	23.2	32.9	53.7	71.3	89.8

Polyporus annosus, where its growth-promoting activity appeared very gradually. In *Trametes heteromorpha* the inhibition was very marked. It is evident, however, that in certain cases this inhibition may change into a growth-promoting effect, as happened in *Polyporus annosus* and probably also in *Polyporus abietinus*.

A greatly increased production of mycelium was obtained with aniline in four species, viz. *Trametes heteromorpha*, *Lenzites betulina*, *Polyporus abietinus*, and *Tricholoma fumosum*, this increase exceeding the control by from 50 to 100 per cent. Interestingly enough, an initial inhibition could be

Table 6. *The effect of tryptophan and aniline on the growth of seven Hymenomycetes.*

Species tested	Time in days	Dry weight of mycelium in mg			
		No further addition	91 μg aniline per flask	456 μg aniline per flask	1000 μg tryptophan per flask
<i>Tricholoma fumosum</i>	15	61.1	74.0	91.5	91.2
	20	177.9	178.7	173.6	201.3
<i>Lentinus lepideus</i>	10	11.1	10.1	10.4	3.4
	20	45.5	46.0	50.0	19.5
<i>Trametes heteromorpha</i>	20	6.1	8.4	5.4	2.4
	30	23.1	53.2	25.0	4.4
<i>Lenzites betulina</i>	10	18.1	29.9	21.8	16.2
	15	93.6	126.9	89.8	79.8
<i>Polyporus abietinus</i>	10	16.2	16.2	14.9	8.1
	20	56.5	74.9	101.9	68.0
<i>Polyporus annosus</i>	20	34.2	35.7	18.7	21.3
	30	67.3	67.3	74.5	109.3
<i>Peniophora fraxinea</i>	20	15.7	14.0	10.5	16.8
	30	41.9	36.6	—	33.4

Table 7. *The inhibiting effect of tryptophan on the growth of Lentinus lepideus.*

μg tryptophan added per flask	Dry weight of mycelium in mg after	
	15 days	25 days
0	15.9	100.0
10	16.8	96.2
30	17.8	103.1
100	16.0	83.3
300	8.5	80.3
1000	7.4	35.3

observed even here, which, however, soon changed to a positive effect, strong in *Polyporus abietinus*, weak in *Polyporus annosus*.

One of these fungi, *Lentinus lepideus*, was further studied with regard to the inhibiting effect of tryptophan (Table 7). At the beginning of the experiment, a suppression of the growth-rate was produced by 300 μg tryptophan per flask. Later on this suppression was overcome, but that obtained with 1000 μg still remained. An interpretation of this effect is proposed in the following discussion.

Discussion

Although *Lentinus omphalodes* is obviously capable of utilizing a nutrient solution with glucose and thiamin as the only organic constituents, the growth-rate obtained under such conditions is far from optimal, particularly in the beginning. With a further addition of tryptophan, however, a rate of growth can be reached, which is equivalent to that obtained with yeast extract or hydrolyzed casein.

A similarly favourable effect of tryptophan has earlier been observed in certain bacteria, *e.g.* *Bacterium typhosum* (Fildes, Gladstone, & Knight 1933, Fildes 1940, 1945, Rydon 1948) and several lactic acid bacteria (Snell, Strong, & Peterson 1937, Snell 1943, Wright & Skeggs 1945, Schweigert, Säuberlich, Baumann, & Elvehjem 1946, Schweigert 1947). In fungi, on the other hand, a requirement for tryptophan or even a growth-promoting effect of this amino-acid has never been noticed, except in the artificially produced »tryptophan-less» mutants of *Neurospora crassa* (Tatum & Bonner 1944, Tatum, Bonner, & Beadle 1944), *Penicillium notatum-chrysogenum* (Bonner 1946), and *Aspergillus nidulans* (Pontecorvo 1950), as well as in *Tricholoma fumosum*, which normally requires tryptophan for maximal growth (Norkrans, unpublished). Investigations have shown that these organisms require tryptophan because they lack the power of synthesizing it them-

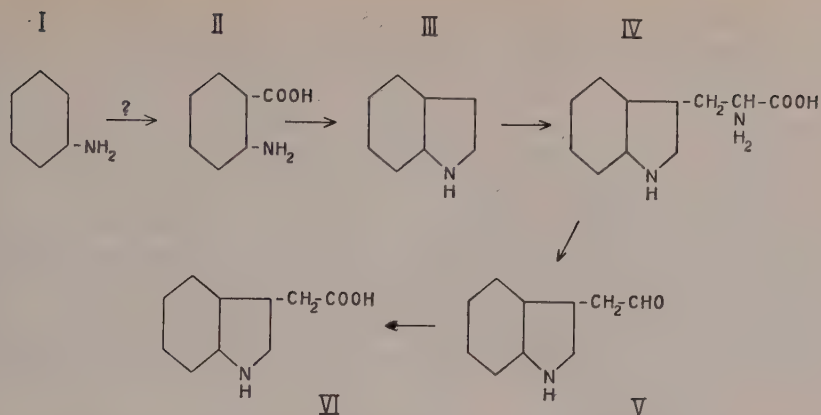


Fig. 1. The sequence of reactions discussed in the text. I: aniline; II: anthranilic acid; III: indole; IV: tryptophan; V: 3-indole acetaldehyde; VI: 3-indoleacetic acid.

selves. This interpretation is certainly the most likely one also in the case of *Lentinus omphalodes*, the loss of synthesizing power in this fungus, however, not being complete, as in most of the above-mentioned cases, but partial.

In several of the bacteria and fungal mutations referred to above it has been found that tryptophan can be exchanged for indole or anthranilic acid. Based upon experiments with bacteria the hypothesis has been advanced that indole (Fildes 1940) and anthranilic acid (Snell 1943) are precursors of tryptophan (Fig. 1). The more recent studies on *Neurospora*-mutants strongly supported this hypothesis, since mutants were found with genetical blocks in different links of the presumed chain of biosyntheses: $x \rightarrow$ anthranilic acid \rightarrow indole \rightarrow tryptophan (Tatum, Bonner, & Beadle 1944, Tatum & Bonner 1944, Nyc, Mitchell, Leifer, & Laugham 1949).

The experiments with *Lentinus omphalodes* showed that this normally tryptophan-requiring fungus also can do well with indole or anthranilic acid. Contrary to the bacteria that utilize anthranilic acid less efficiently (Snell 1943) *Lentinus* responded equally well to all three substances. Of other indole-derivatives tested, only 3-indolebutyric acid produced a significant growth-promoting effect, much weaker, however, than that of indole; 3-indolepropionic acid was inactive, and 3-indoleacetic acid (heteroauxin), as could be expected, strongly inhibiting.

The clearly positive effect of aniline appears, however, to be the most interesting result of the experiments with aromatic, non-indole compounds. The effect is the more surprising as the substance in question has been regarded chiefly as a toxic agent, used as an antiseptic (Huntington & Rahn

1945, Frear 1948) and a fixative (Unat 1942). When tested earlier in experiments with tryptophan-deficient organisms, aniline proved to be entirely inactive (Tatum, Bonner, & Beadle 1944, Schweigert et al. 1946). Being a substance of even more simple constitution than anthranilic acid, aniline can be assumed to represent a precursor of anthranilic acid in the indole synthesis. This does not, of course, necessarily mean that aniline should be the normal natural precursor, all the more as aniline has not so far been shown to occur as a metabolite in fungi. The possibility should also be considered that aniline is more directly transformed into indole (or tryptophan), since Nyc et al. (1949) have found in *Neurospora*, by means of isotopic technique, that the carboxyl carbon of anthranilic acid is not present in the formed tryptophan.

If a comparison is made on a molar basis, aniline is evidently less active than anthranilic acid, indole, and tryptophan, its optimal concentration being higher than those of the latter substances. If the maximal possible effects are compared, aniline is about as active as the others. On the other hand, indole appears to be more toxic than aniline.

All other substances tested because of their structural relations to indole or anthranilic acid, exhibited no growth-promoting activity, except 3-indolebutyric acid, and perhaps also o-toluidine and isatin, the effects of which were not statistically significant. The negative result with nicotinamide seems to show that even if this vitamin is formed from tryptophan, which is obviously the case in some fungi (see Beadle, Mitchell, & Nyc 1947, Pontecorvo 1950), the reverse reaction is not possible in *Lentinus omphalodes*.

The fact that *Lentinus omphalodes* is not absolutely dependent on tryptophan or its precursors makes it very difficult to prove, without tracer experiments, that aniline is actually involved in the tryptophan-synthesis. A certain indirect evidence may be obtained by comparing the response of different species to the substances in question. From the preliminary experiment so far performed with a few other fungi, it appeared that aniline promoted growth in the same cases as did tryptophan. However, a positive effect was often produced by aniline in fungi, where tryptophan was quite inactive or even inhibitory.

This growth-inhibiting effect of tryptophan was evident in at least four of the seven Hymenomycetes investigated. It might perhaps be explained as a conversion of tryptophan to the phytohormone, 3-indoleacetic acid, which has a well-known inhibiting effect on fungal growth (Leonian & Lilly 1937, Murdia 1939, Bhargava 1946, Richards 1949). This conversion, including 3-indole acetaldehyde as an intermediate, is readily performed in plants (see *i.a.* Thimann 1935, Larsen 1944, Gordon & Nieva 1949). According to this tentative interpretation, the degree of tryptophan-inhibition should depend,

firstly, upon the velocity by which the tryptophan is converted to 3-indoleacetic acid; secondly, upon the sensitivity of the fungus in question to this latter substance (Fig. 1).

Summary

The Hymenomycete, *Lentinus omphalodes*, was shown to require an external supply of tryptophan to produce a maximal rate of growth. The same effect is obtained with anthranilic acid and indole, both of which have been earlier found (in studies on bacteria and fungal mutations) to represent intermediates in the biosynthesis of tryptophan. A strong positive effect was also produced by aniline, which might indicate that this substance can serve as a precursor of tryptophan.

A positive response to aniline was observed also in a number of other Hymenomycetes. The inhibiting effect of tryptophan found in some cases was tentatively interpreted as caused by a conversion of this amino-acid to 3-indoleacetic acid.

The author is greatly indebted to Miss Anna Panders for skilful technical assistance.

References

- Beadle, G. W., Mitchell, H. K., & Nyc, J. F.: Kynurenine as an intermediate in the formation of nicotinic acid from tryptophane by *Neurospora*. — *Proc. Nat. Acad. Sci. U. S.*, 33: 155. 1947.
- Bhargava, K. S.: Physiological studies on some members of the family Saprolegnicaceae. V. Growth substances. — *Lloydia*, 9: 13. 1946.
- Bonner, D.: Production of biochemical mutations in *Penicillium*. — *Amer. Journ. Bot.* 33: 788. 1946.
- The identification of a natural precursor of nicotinic acid. — *Proc. Nat. Acad. Sci. U. S.*, 34: 5. 1948.
- Cushing, J. E., Schwartz, M., & Bennett, R.: Altered responses of *Neurospora crassa* to inhibiting concentrations of indole. — *Journ. Bact.*, 58: 433. 1949.
- Fildes, P.: Indole as a precursor in the synthesis of tryptophan by bacteria. — *Brit. Journ. Exper. Pathol.*, 21: 315. 1940.
- The biosynthesis of tryptophan by *Bacterium typhosum*. — *Brit. Journ. Exper. Pathol.*, 26: 416. 1945.
- Fildes, P., Gladstone, G. P., & Knight, B. C. J. G.: The nitrogen and vitamin requirements of *B. typhosus*. — *Brit. Journ. Exper. Pathol.*, 14: 189. 1933.
- Frear, D. E. H.: A catalogue of insecticides and fungicides. II. Chemical fungicides and plant insecticides. — Waltham, Mass. 1948.
- Fries, N.: Effects of different purine compounds on the growth of guanine-deficient *Ophiostoma*. — *Physiol. Plant.*, 2: 78. 1949.
- Growth factor requirements of some higher fungi. — *Svensk Bot. Tidskr.*, 44 (in press). 1950.

- Gordon, S. A. & Nieva, F. S.: Biosynthesis of auxin in vegetable pineapple. II. Precursors of indolylacetic acid. — Arch. Biochem., 20: 366. 1949.
- Huntington, G. I. & Rahn, O.: Acidity controlling antiseptics by weak acids. — Journ. Bact., 50: 655. 1945.
- Larsen, P.: 3-indole acetaldehyde as a growth hormone in higher plants. — Dansk Botanisk Arkiv, Vol. 11. No. 9: 1. 1944.
- Leonian, L. H. & Lilly, V. G.: Is heteroauxin a growth-promoting substance? — Amer. Journ. Bot., 24: 135. 1937.
- Murdia, M. S.: Effect of β -indolyl 3-acetic acid and phenyl acetic acid on the growth of some members of the family Saprolegniaceae. — Current Science, 8: 362. 1939.
- Nyc, J. F., Mitchell, H. K., Leifer, E., & Langham, W. H.: The use of isotopic carbon in a study of the metabolism of anthranilic acid in Neurospora. — Journ. Biol. Chem., 179: 783. 1949.
- Pontecorvo, G.: New fields in the biochemical genetics of micro-organisms. — Biochemical Society Symposia No. 4 (Biochemical aspects of genetics): 40. Cambridge, 1950.
- Richards, R. R.: Responses of representative fungi to certain growth-regulating substances. — Bot. Gaz., 110: 523. 1949.
- Rydon, H. N.: Anthranilic acid as an intermediate in the biosynthesis of tryptophan by Bact. typhosum. — Brit. Journ. Exper. Pathol., 29: 48. 1948.
- Schweigert, B. S.: The role of vitamin B₆ in the synthesis of tryptophane from indole and anthranilic acid by Lactobacillus arabinosus. — Journ. Biol. Chem., 168: 283. 1947.
- Schweigert, B. S., Säuberlich, H. E., Baumann, C. A., & Elvehjem, C. A.: The tryptophane activity of various compounds for L. arabinosus and their influence on the determination of tryptophane in natural materials. — Arch. Biochem., 10: 1. 1946.
- Snell, E. E.: Growth promotion on tryptophan-deficient media by o-aminobenzoic acid and its attempted reversal with orthanilamide. — Arch. Biochem., 2: 389. 1943.
- Snell, E. E., Strong, F. M., & Peterson, W. H.: Growth factors for bacteria. VI. Fractionation and properties of an accessory factor for lactic acid bacteria. — Biochem. Journ., 31: 1789. 1937.
- Tatum, E. & Bonner, D.: Indole and serine in the biosynthesis and breakdown of tryptophane. — Proc. Nat. Acad. Sci. U. S., 30: 30. 1944.
- Tatum, E. L., Bonner, D., & Beadle, G. W.: Anthranilic acid and the biosynthesis of indole and tryptophan by Neurospora. — Arch. Biochem., 3: 477. 1944.
- Thimann, K. V.: On the plant growth hormone produced by Rhizopus suinus. — Journ. Biol. Chem., 109: 279. 1935.
- Unat, E. K.: Über die Resistenz embryonierter Ascariseier. — Istanbul Univ. Tib. Fakültesi Mecmua, 5: 21. 1942.
- Wright, L. D. & Skeggs, H. R.: Tryptophane utilization and synthesis by strains of Lactobacillus arabinosus. — Journ. Biol. Chem., 159: 611. 1945.

Methylene Blue and α - α' -dipyridyl, two Different Types of Inhibitors for Aerobic Metabolism in Young Wheat Roots

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1. Introduction and methods

Inhibition of respiration in plant roots also implies inhibited salt absorption. Lundegårdh (for a review see Lundegårdh 1945 and 1950) has shown that inhibition of a special part of the respiration process totally stops salt absorption. This part of respiration amounts to cirka 60—70 % of normal respiration and is inhibited by HCN, NaN_3 and CO (with light reversal; see Sutter 1950). These facts support the view that an iron enzyme, probably cytochrome oxidase, is the terminal oxidase in this special respiration. In this paper some experiments with dipyridyl (DP), a chelating agent specific for divalent iron will be reported as a contribution to this question.

In the case of many inhibitors, the absorption of ions is inhibited at lower concentrations than is the respiration. In some cases it is possible to stop the absorption of ions without any decrease in the rate of respiration. Hoagland and Broyer (1942) observed that in the presence of methylene blue (MB) the absorption of bromide was inhibited although the CO_2 production was normal. They used only rather high and immediately toxic concentrations of MB (10^{-3} M) however, and in the present paper some data will be given about the effect of lower concentrations of MB upon oxygen consumption and the absorption of chloride and glucose.

The experiments were performed with excised wheat roots, 3—4 days old and 30—40 mm long. For further details of the determinations of O_2 consumption and glucose absorption see Stenlid 1949. The chloride absorption was determined by electrometric titrations with AgNO_3 of the chloride content in the surrounding solu-

tion. The solution was aerated throughout the experiment and about 75 mg dry matter in 50 ml of solution was used in each experiment. The determinations of the absorption of chloride were first made in pure solutions of KCl. However, as the pH during these experiments fell towards 4.5, experiments were also made in a 0.002 *M* KCl solution that was 0.001 *M* with respect to phosphate buffer of pH 7. The inhibitors affected the chloride absorption in the same way in the solutions with and without phosphate. The absolute amount of chloride absorbed by the controls without inhibitor was about 3.5 μ gions/hour and 100 mg dry matter. All experiments were made at 25° C.

2. Experiments with α - α' -dipyridyl

Several inhibitors (e.g. HCN, NaN_3 , and CO) have been applied to clarify the relationship between respiration and the absorption of ions. As mentioned above, the experiments with these inhibitors indicate that about two thirds of the respiration in wheat roots is of a special kind and catalysed by an iron enzyme.

None of these inhibitors, however, is specific for iron (the light reversibility with CO is often difficult to obtain) and therefore some experiments with

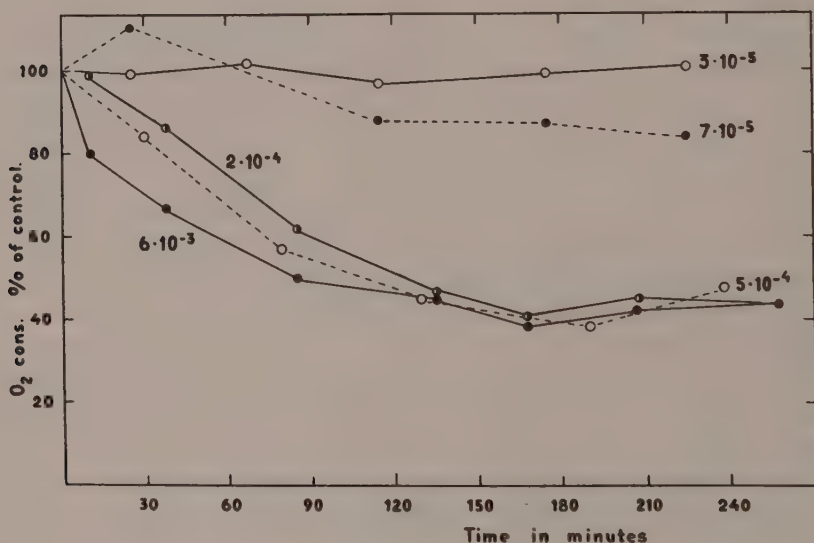


Fig. 1. The effect of different concentrations of DP upon oxygen consumption in young wheat roots in 0.01 *M* phosphate buffers of pH 7.0. Determinations made in a Warburg-Barcroft apparatus. Volume of buffer 1.0 ml; 0.2 ml inhibitor added. 15 root tips (15 mm long) in each vessel. The values give the O₂ consumption in buffer+inhibitor expressed as % of the consumption in pure buffer solutions. Every value is the mean from 2—3 vessels with control and 2—3 vessels with inhibitor.

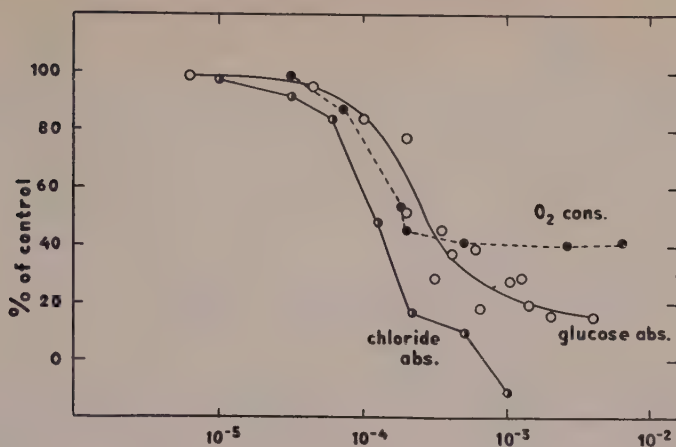


Fig. 2. The effect of DP upon O_2 consumption, glucose absorption and chloride absorption in young wheat roots. O_2 consumption measured 120–180 minutes after the addition of the inhibitor; the values are taken from experiments similar to those in fig. 1. The glucose absorption was determined with 30–40 mm long roots. 0.005 M glucose solutions with phosphate buffer of pH 7 were used. For further details see Stenlid 1949. The chloride absorption was determined with roots of the same length (for further details see page 197). Duration of the absorption experiments 5 hours. Every value is the mean from 2–3 determinations. — On the abscissa conc. in mol./l.

DP can be of interest. Albert & Gledhill (1947) showed that under physiological conditions (pH 7, water solutions) DP is a quite specific chelating agent for divalent iron. It is sufficiently water soluble and has about the same effect upon wheat roots at pH 4.5 and pH 7.0. The effect of DP upon O_2 consumption, glucose absorption and chloride absorption is shown in figures 1 and 2.

The results strongly indicate that about 60 % of the oxygen uptake is regulated by a special mechanism with iron as the necessary metal. Inhibition of this 60 % with DP stops the absorption of chloride ions and of glucose. All inhibitions run rather parallel. The exudation (exosmosis) of reducing substances into phosphate buffers with different concentrations of DP was also determined and is given in fig. 3. It is seen that no appreciable exudation begins until the concentration of DP is so high that the maximum inhibition of O_2 uptake is already obtained. Thus it can be concluded that the primary effect of DP (visible in very dilute solutions) is an inhibition of respiratory enzymes and thereby the absorption of chloride ions and glucose, whereas the effects giving exudation are secondary and visible only in more concentrated solutions.

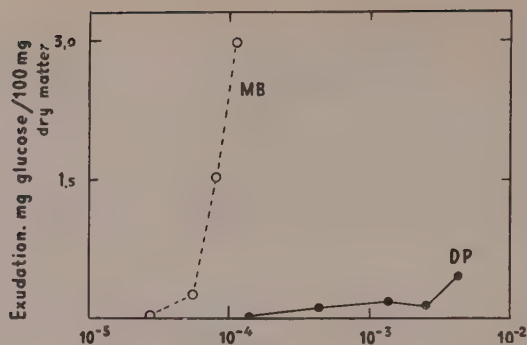


Fig. 3. The exudation of reducing groups (calculated as glucose; determined according to Philipson 1943) from wheat roots (excised roots 30—40 mm long) in phosphate buffers with different concentrations of MB and DP. About 70 mg dry matter in 50 ml of solution was used for each determination. Every value is the mean of 2—3 determinations. Duration of the experiments 5 hours, pH 7.0. — On the abscissa conc. in mol./l.

3. Experiments with methylene blue. Discussion

The effects of different concentrations of MB upon O_2 consumption, chloride absorption, glucose absorption, and the exudation of reducing substances were determined in the same way as for DP. The results are given in figures 3 and 4. Also MB has about the same effect at pH 4.5 and pH 7.0.

As in the experiments with DP, all the processes are affected but there are obvious differences in the relative strength of the effects. With MB, the absorption of glucose and chloride is inhibited already at concentrations where the O_2 consumption is quantitatively normal or even somewhat increased. A possible explanation is that a new type of respiration is induced and that the normal respiration is at the same time inhibited. However, the sensitivity of O_2 consumption to DP and ortho-phenanthroline after addition of MB is only slightly changed, which means that it is most probable that the cytochrome oxidase system is still active.

The exudation of reducing substances begins at concentrations where no inhibition of the oxygen uptake is noticeable. It was also possible to determine the exudation of substances absorbing ultra-violet light. The ultra-violet absorption of the surrounding solution was measured with a Beckman spectrophotometer and the ultra-violet absorption corresponding to the unabsorbed MB was subtracted (the concentration of MB not absorbed by the roots was calculated from the absorption of red light). As in the case of reducing substances an obvious increase in the exudation of ultra-violet absorbing substances was observed at an initial MB concentration of cirka

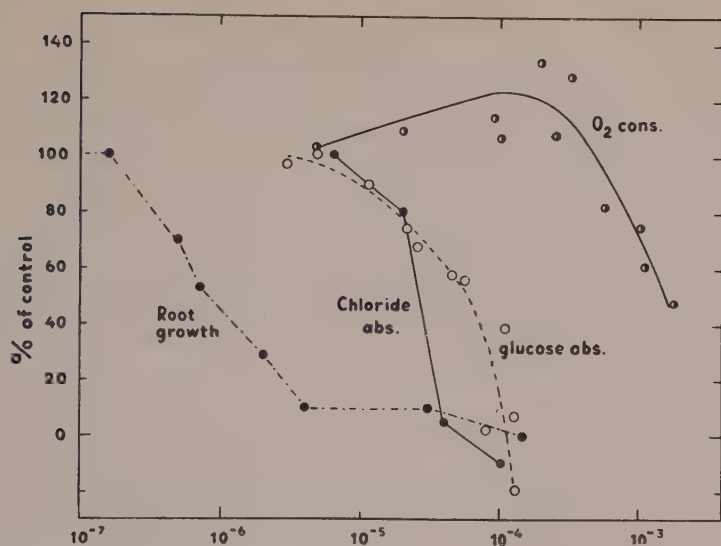


Fig. 4. The effect of MB upon root growth, O_2 consumption, glucose absorption and chloride absorption in young wheat roots. For further details see fig. 2 and page 202.

$5 \cdot 10^{-5}$ M. In the experiments with MB, the inhibitory effect upon O_2 consumption seems to be a secondary phenomenon and is not caused by a direct attack upon the respiratory enzymes. MB in low concentrations has been shown to disturb phosphorylation and phosphorus transport (Judah & Williams-Ashman 1949) and it seems probable that this leads to partial injuries and disorganisation in the protoplasm. The exudation is thus increased and the absorption of glucose and chloride inhibited. When the injury has proceeded beyond a certain limit the respiration also must be affected. The effect of MB strongly resembles that given by 2,4-dinitrophenol (see Stenlid 1949) which is also known to have a marked effect on phosphorylation processes.

The absorption of glucose and the exudation of reducing groups show a different sensitivity to DP, the absorption being more sensitive. On the other hand, if MB or 2,4-dinitrophenol is applied it is not possible to distinguish between these two processes as it seems probable that for these inhibitors the same inhibitory mechanism (inhibition of phosphorus metabolism) is responsible both for the decreased glucose absorption and the increased exudation of reducing groups.

There is no obvious difference between the concentrations of DP inhibiting the absorption of glucose and of chloride ions. This is also true for MB. 2,4-dichlorophenoxyacetic acid (2,4 D) in low concentrations stops the

absorption of anions in excised wheat roots (Nance 1949) whereas the glucose uptake is only slightly affected (see Stenlid 1949). The experiments with MB and DP indicate that the accumulatory mechanisms for chloride and glucose are similar in some respects but the results from the experiments with 2,4-D suggest that there is also a distinct difference. The experimental conditions in the two investigations with 2,4-D, however, were not identical and further experiments are necessary.

In fig. 4 are included some data on the effect of MB upon longitudinal root growth. Since these data were obtained with intact wheat plants in nutrient solutions, the duration of the experiments being 6 days, they are not directly comparable with the other data in the same figure. They are only given to show that low concentrations of MB are strongly toxic for higher plants. It should be stressed that the concentrations given are concentrations in the surrounding medium and that the concentrations inside the root are much higher. Especially in the extreme root tip (3—4 mm) a pronounced accumulation of MB takes place rapidly. Guillermond & Gautheret cultivated wheat in Knop's solution with gelose and they found higher concentrations of MB necessary to give strong inhibitions of root growth than those effective in the water cultures used in the present experiments.

Summary

The effect of methylene blue (MB) and α - α' -dipyridyl (DP) upon oxygen consumption and the absorption of chloride ions and glucose by excised wheat roots has been studied. The exudation of reducing substances and in the case of MB also root growth and exudation of ultra-violet absorbing substances was determined.

DP in low concentrations ($2.5 \cdot 10^{-4}$ M) inhibits 60 % of O_2 consumption. No further inhibition is obtained in $7 \cdot 10^{-3}$ M solutions. Inhibition of this 60 % of O_2 consumption gives almost total inhibition of the absorption of chloride ions and of glucose. The primary effect of DP seems to be inhibition of some iron-containing respiratory enzyme. Exudation of reducing substances is obvious first in solutions $> 2 \cdot 10^{-3}$ M.

MB in low concentrations (10^{-4} M) inhibits the absorption of glucose and chloride ions. This concentration also causes strong exudation of reducing substances and of substances absorbing ultra-violet light. Concentrations of $> 5 \cdot 10^{-4}$ M are needed to inhibit O_2 consumption. The primary effect of MB seems to be inhibition of the phosphorus metabolism. Root growth in water cultures is inhibited to 50 % by MB in a concentration of 10^{-6} M.

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References

- Albert, A. & Gledhill, W. S.: The choice of a chelating agent for inactivating trace metals. I. A survey of commercially available chelating agents. — *Bioch. J.* 41: 529. 1947.
- Guillermont, A. & Gautheret, R.: Recherches sur la coloration vitale des cellules végétales. — *Rev. Gén. Bot.* 52: 353, 440. 1940. 53: 25, 79, 121, 158, 212, 315, 362. 1946.
- Hoagland, D. R. & Broyer, T. C.: Accumulation of salt and permeability in plant cells. — *J. Gen. Physiol.* 25: 865. 1942.
- Judah, J. D. & Williams-Ashman, H. G.: Some inhibitors of aerobic phosphorylation. — *Bioch. J.* 44 (4): XL (*Proc. Bioch. Soc.*). 1949.
- Lundegårdh, H.: Absorption, transport, and exudation of inorganic ions by the roots. — *Arkiv f. Bot. Stockholm*, Bd 32 A. N:o 12. 1945.
- The translocation of salts and water through wheat roots. — *Physiol. Plant.* 3: 103. 1950.
- Nance, J. F.: Inhibition of salt accumulation in excised wheat roots by 2,4-dichloroacetic acid. — *Science* 109: 174. 1949.
- Philipson, T.: Über die quantitative Bestimmung reduzierender Zucker. — *Arkiv f. Kemi, Miner. och Geol. Stockholm*. Bd 16 A. N:o 22. 1943.
- Stenlid, G.: The effect of 2,4-dinitrophenol upon oxygen consumption and glucose uptake in young wheat roots. — *Physiol. Plant.* 2: 350. 1949.
- Sutter, E.: Über die Wirkung des Kohlenoxydes auf Atmung und Ionenaufnahme von Weizenwurzeln. — *Experientia*. 1950. (in print).

Further Studies on the Growth of Isolated Roots of Barley and Oats

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In an earlier paper (2) some studies on the growth of isolated roots of barley and oats were described. In spite of widely varied culture media these roots were unable to grow indefinitely, a result also found by the cultivation of roots of several other species of monocotyledonous plants (6). Microscopical examination showed that the cessation of growth was caused by inactivity of the apical root meristem. The results indicated that in the endosperm and leaves of monocotyledons an unknown substance is synthesized, which is necessary for the meristematic cell divisions. This substance is not identical with any of the general accessory growth substances.

In this connexion it should be mentioned that, in the infinite cultures of tomato roots described in the literature, subcultures apparently start from laterals in most cases it seems to be undecided whether one apical meristem is actually capable of unlimited growth. As regards pea roots, however, the original meristem is transferred by subculturing, and thus these roots seem to have an unlimited growth. There are, consequently, some reasons for suspecting that even the growth factors of isolated roots of dicotyledons are not yet completely solved.

Very different views upon the growth of roots are to be found in the literature. Thus Padmasini (15) discussed some experiments with roots of *Eleusine coracana*, saying that these roots could grow in a basic medium of sucrose and inorganic salts. The duration of the experiments was 5 days, and, as the roots elongated during this time, Padmasini stated that the investigation had shown that the roots were able to synthesize their necessary vitamins. According to the author's observations on his material such growth

is generally obtained if the medium is not extraordinarily unsuitable, and it is caused by elongation of meristem cells. For such a short time roots can grow without any addition of vitamins.

This paper contains the results of some continued investigations with the aim of studying the causes of the inability of grass roots to grow indefinitely. The experiments are divided into 3 groups:

1. Effects of the method of sterilization.
2. Effects of plant extracts.
3. Effects of some nitrogen compounds.

Methods

The experimental material employed was the same as earlier, namely, roots of barley (Weibull's »Herta») and oats (Weibull's »Bambu»). The method of sterilizing the seeds was the one already described. As regards the inoculation some details must be added here. The scissor and the forceps were sterilized over a gas flame and not according to the method described by White (25). Some control experiments have shown, however, that this difference of methods had no decisive effect. After excision the root tips (length 10 mm.) were transferred into a beaker containing sterile water and then from here into the culture vessels by means of forceps and not by a platinum loop. The basic medium has been the same as in previous investigations. Every treatment has included 5 parallel cultures of 4 roots. The duration of each experiment has been 10 days, and the total lengths of the roots were used as a measure of the growth. Generally about 10 per cent, sometimes more, of the roots of every treatment grew very poorly or stopped completely. It is assumed that the roots had been injured by the excising or transferring. If such roots were excluded the mean error in root length expressed in per cent of the average value amounted to about 10 per cent.

The roots were also subjected to a microscopical examination.

Results

1. *Effects of the method of sterilization*

a. Sugars

In the previous experiments the media were always sterilized by autoclaving generally at 110° C for 20 minutes. Even if most of the substances of the basic medium are generally regarded as thermostable, there is, how-

ever, some reason for suspecting that decomposition can take place by autoclaving the complete solution. Thus it is possible that by autoclaving substances can be formed, disturbing the normal metabolism of the roots, and in this manner causing the cessation of meristem cell divisions. However, it is possible to avoid autoclaving by using sterile filtration of the nutrient media. This method is somewhat more troublesome and as far as the author is aware, it has been used very infrequently in investigations of this type (cf. McClary 13, however). Solomon (23) found that autoclaved malt extract had an inhibitory effect upon the growth of *Datura* embryos. A new compound was formed, masking the effect of the embryo growth factor. Such an effect could be eliminated, when the extract or nutrient media containing extract was sterilized by filtering in a Seitz filter. In the literature (cf. White 25) it is often stated that glucose is decomposed by autoclaving, and Smith (22) has made some experiments indicating that if a nutrient solution containing 3 gm. of glucose per litre is heated for 40 min. at 15 lbs. at pH 8 (phosphate conc. 0.8 per cent) 30 per cent of the sugar disappears. White (25) states that sucrose is heat-stable, but Burström (7) has proved that sucrose in a common nutrient solution is hydrolyzed to a great extent already by autoclaving for 20 min. at 110° C, a rather low heating. Thus the statements are often contradictory. Very little is known about the nature of the decomposition products of glucose.

The following filters are available for sterile filtration: a) Seitz filter, b) Berkefeld filter, c) Chamberland filter, d) Pyrex glass filter H 5 and e) Membrane filter after Zsigmondy. As regards, a, b, and c there are investigations showing that large quantities of substances are adsorbed in the filters (cf. Fries 9, who found that thiamin was adsorbed in a Seitz filter). These filters, therefore, cannot be used for filtration, as the accessory substances will disappear from the filtrate. The most suitable type of filters are membrane filters after Zsigmondy. These are thin, have small adsorption capacity and can easily be changed. In the experiments given below membrane filters »feinst», ø 40 mm. with the average pore diameters of 20—100 m μ have been used. The filters are made by Membranfilter-Gesellschaft, Göttingen, Germany. For the filtration a so-called Thiessen's apparatus for vacuum and pressure filtration has been used. All filtration were vacuum filtrations. The apparatus with filter and suction flask were autoclaved and in order to avoid a drying of the filter and the subsequent cracking, some ml. water were poured into the filter funnel. The nutrient media were supplied automatically and each filter could generally be used 3 or 4 times.

By the normal autoclaving of the nutrient solution in my experiments, there was generally a pH decrease from 5.8—6.0 in newly prepared solution to 4.6—4.8. Such a decrease was not obtained immediately in sterile filtration

Table 1. *The concentrations of glucose (mmol per 20 ml.) in nutrient solutions sterilized in different manners. (Average values of 4 determinations.)*

Untreated basic medium		Sterilized basic medium	
Theoretical conc.	Determined conc.	Autoclaving for 20 min. at 110°C	Sterile filtration
0.400	0.366	0.361	0.360

Table 2. *The final lengths in mm. of roots of barley and oats cultivated in autoclaved and sterile filtered media.*

Sterilization method	Autoclaving	Sterile filtration
Barley	34	34
Oats	25	36

but appeared after about 2 days and probably depended upon dissolved carbon dioxide. Thus the increased hydrogen ion concentration does not seem to have been caused by the acid decomposition products of glucose formed.

A few determinations of the glucose concentrations in solutions treated in different manners have been made. The theoretical concentration was 1/20 mol per litre and the determinations were carried out according to a method described by Philipson (16) (Table 1).

Thus the glucose concentration decreased somewhat more than 1 per cent both by sterile filtration and autoclaving. It was not characteristic, however, that a special decomposition took place in autoclaving.

A growth experiment with autoclaved and sterile filtered media gave the following results (Table 2).

No differences could be observed in the length of barley roots but the oats roots became shorter in the autoclaved solution. The microscopical examination showed that the roots in both types of media were tapering and had only a few non-elongated meristem cells, oats, however, more than barley. Previous observations (2) also showed this phenomenon that the meristem cells of barley elongated more rapidly than those of oats. A measurement of the meristem residues of oats gave the following results: in autoclaved solution 0.43 mm. and in sterile filtered 0.31 mm. Thus a smaller number of meristem cells had elongated in the autoclaved solution than in the sterile filtered one.

In order to study more closely the exact effects of the time and pressure

Table 3. *The root length in mm. and the number of laterals of barley and oats cultivated in media with varied autoclaving of the glucose solution.*

Sterilization method		Sterile filtration	Autoclaving of the glucose solution		
			For 20 min. at 1 atm.	For 20 min. at 2 atm.	For 60 min. at 2 atm.
Barley	{Length	31	40	39	38
	{Laterals	2,9	3,5	3,6	3,6
Oats	{Length	34	35	33	33
	{Laterals	0	0	0	1

Table 4. *The root lengths in mm. of barley and oats cultivated in nutrient solutions of varied glucose concentration and varied heating of the glucose.*

Glucose conc.	1/50 mol		1/20 mol	
Sterilization method	Autoclav. for 20 min. at 110°C	Sterile filtration	Autoclav. for 20 min. at 110°C	Sterile filtration
Barley	31	29	27	32
Oats	28	29	33	30

of autoclaving, some experiments were performed with culture media prepared as follows. The glucose solutions were heated separately and the time and pressure were varied. Then each such solution was mixed with mineral and vitamin solutions, and the mixtures were sterile-filtered. In this way the effects of the minerals and vitamins ought to be rather uniform and eventually obtained variations in growth must be ascribed to the sugar component (Table 3).

The root length of barley in sterile-filtered solutions was smaller than in the autoclaved ones, which were uniform among themselves. The oat roots had the same lengths in all solutions. The number of laterals was about the same in all treatments of each species, but as is generally the case the laterals of oats were fewer than those of barley. The meristem cells had elongated similarly in both series. Thus no differences caused by varied duration of autoclaving could be noted.

It would also be of interest to see, if glucose solutions of different concentrations were influenced in various manners by autoclaving (Table 4).

The root lengths were uniform and a microscopical examination showed no differences within both series, but some meristem residues could be detected in the oat roots.

In summarizing it may be stated that the recorded experiments showed

Table 5 a. *A comparison of 3 experiments on the effects of varied autoclaving on root length and glucose absorption of roots of barley and oats. Lengths are given in mm. and glucose absorption in mmol per 20 roots.*

Sterilization method		Only glucose solution autoclaved		Glucose and minerals autoclaved		Total solution autoclaved	
Species		Barley	Oats	Barley	Oats	Barley	Oats
Sterile filtration	Absorbed glucose	0.070	0.016	0.068	0.023	0.070	0.026
	Root length	42	22	34	33	33	18
Autoclaving 60 min. at 1 atm.	Absorbed glucose	0.072	0.019	0.073	0.031	0.064	0.012
	Root length	42	23	31	26	29	21
Autoclaving 60 min. at 2 atm.	Absorbed glucose	0.092	0.045	0.076	0.023	0.056	0.030
	Root length	40	19	44	32	32	19

Table 5 b. *The glucose absorption and lengths of roots of barley and oats cultivated in media with the glucose component autoclaved at varied pH. Lengths are given in mm. and glucose absorption in mmol per 20 roots.*

Species	Barley	Oats	pH
Absorbed glucose	0.092	0.031	4
Root length	47	22	
Absorbed glucose	0.066	0.023	6
Root length	45	25	
Absorbed glucose	—	0.038	8
Root length	30	18	

that no toxic compounds, inhibiting normal root growth, were formed by the normal autoclaving of the media.

Carbohydrates, generally sucrose or glucose, must be added to the nutrient solution for excised roots, and the roots elongate under a sugar absorption, smaller or greater according to the rate of growth. It could be suspected that the sugar absorption would vary in solutions sterilized in different ways and for elucidating this problem some investigations on root growth and the glucose absorption connected with this were performed. The results are given in Tables 5 a and b. Every experiment was repeated in 4 parallel cultures with 20 root tips in each culture vessel. The concentration of glucose was 1/50 mol per litre. The values of root lengths are average values of 60—80 roots and the glucose absorption was estimated as the average value of 3—4 cultures each with 2 agreeing determinations. The investigation was performed in 4 different sections, namely:

1. Only the glucose solutions were autoclaved; sterile-filtration after the addition of minerals and vitamins.

Table 6. *Root lengths in mm. of barley and oats cultivated in sucrose media sterilized in different manners. Sucrose conc. 1/20 mol.*

Sterilization method	Sterile filtration	Autoclaving		
		20 min. at 0.5 atm.	60 min. at 1 atm.	60 min. at 2 atm.
Barley	40	58	49	39
Oats	39	45	37	36

2. The glucose solutions with minerals were autoclaved; sterile-filtration after the addition of vitamins.
3. The glucose solution, mineral and vitamins were autoclaved together.
4. The glucose solutions of different pH-values were autoclaved separately at 2 atm. for 60 min. Sterile filtration after the addition of minerals and vitamins. The pH-values of the glucose solutions were varied by the additions of phosphate buffer solutions of a mixture of H_3PO_4 and Na_3PO_4 in such concentration that the phosphate concentration of the total nutrient media would be 1 mmol per litre. The solution of pH 8 assumed a brown colour in autoclaving. The buffer solutions were so weak, that when the nutrient media had been sterile-filtered, the pH-values of the 3 different solutions were about the same, namely 4.3 in the solutions autoclaved at pH 4 and 6 and 4.6 in the solution autoclaved at pH 8. Thus it appears that there is no or little effect of the hydrogen ion concentration on the root growth.

The results showed that in all cases the roots of barley had a larger absorption of glucose than those of oats, about twice as much. For the rest, however, it was difficult to find significant differences in the absorptions in solutions heated in different ways. The same fact was also established concerning the root lengths and no radical differences in the appearances of the roots could be detected. Thus the results indicated that no factor of importance for root growth and glucose absorption was to be found in the methods of sterilization.

Generally sucrose is used in isolated root cultures of dicotyledons and glucose in cultures of monocotyledons. My earlier experiments had shown that growth was somewhat less in sucrose than in glucose. No studies have been made for determining the degree of hydrolysis in the solutions, but some cultures were made with sucrose and fructose autoclaved differently (Tables 6 and 7).

The sucrose solutions were autoclaved separately and sterile-filtered after the addition of minerals and vitamins. The longest roots of both barley and

Table 7. *Root lengths in mm. of barley and oats cultivated in fructose solutions sterilized in different manners. Fructose conc. 1/20 mol.*

Sterilization method	Sterile filtration	Autoclaving		
		20 min. at 0.5 atm.	60 min. at 1 atm.	60 min. at 2 atm.
Barley	36	29	36	36
Oats	25	29	27	30

oats were obtained in the slightest autoclaved medium. It is assumed that some hydrolysis had taken place, and the results depended upon the glucose formed. By stronger heating toxic compounds may be formed. The roots showed no differences, when examined microscopically.

The growth in fructose was less than in both glucose and sucrose, and the number of malformed roots was extraordinarily great. The results agreed with Burström's (7), who found that wheat roots grow normally in fructose but more slowly than in glucose.

b. Accessory growth factors

In the previous paper (2) it was reported that no effects had been obtained on these root cultures by the addition of two substances, which generally are regarded as thermostable, namely indole acetic acid and thiamin. As is well-known, the former induces cell divisions in plant tissue cultures and the latter is necessary for indefinite growth of most isolated roots of dicotyledons, also by activating the meristems. It could, however, be assumed that these substances did not endure autoclaving without decomposition, and so some experiments were made with sterile filtered media in comparison with autoclaved ones. The heteroauxin concentration was varied from 10^{-18} to 10^{-6} mol per litre (Table 8).

Only a slight promoting effect of heteroauxin could be detected and thus this substance was without any effect on the meristematic activity of these roots if added in normal quantities, in sterilization by autoclaving or by sterile-filtration. In high concentrations the general inhibitory effect appeared.

In this connexion an experiment reported by Bonner (3) may be mentioned. He had discovered that sodium arsenate inhibited the effect of heteroauxin on *Avena coleoptiles* without affecting the respiration. Supposing the roots normally contain an excess of auxin, as is generally the case, an addition of arsenate probably would inhibit its effect and root growth would increase (Table 9).

No results, however, could be detected either on root lengths or on root meristems. Thus the poor growth of these kinds of roots did not seem to be affected by a surplus of auxins.

Table 8. *Root lengths in mm. of barley and oats cultivated in solutions with varied concentrations of heteroauxin and sterilized by autoclaving or sterile filtration.*

Conc. of heteroauxin mol/l.		0	10^{-18}	10^{-16}	10^{-14}	10^{-12}	10^{-10}	10^{-8}	10^{-6}
Sterile filtration	Barley	33	—	33	31	35	32	30	22
	Oats	32	—	37	34	39	42	23	14
Autoclaving	Barley	36	33	39	38	36	35	25	25
	Oats	32	36	30	25	29	28	21	14

Table 9. *The effects of sodium arsenate on root length of isolated roots of barley and oats.*

Sodium arsenate mg/l.		0	1	3	10	30	100
Root length mm.	Barley...	34	35	32	32	30	15
	Oats.....	30	31	33	25	23	15

Also in the investigations with thiamin the root growth in parallel cultures with autoclaved and sterile-filtered solutions was compared. The concentration was varied from 0.1 to 10,000 mg. per litre. In some cultures very long barley roots were obtained, but a study of their meristems indicated no effects. These experiments stated that thiamin is not destroyed by autoclaving and, thus it cannot have the same importance for the roots of monocotyledons as for the roots of dicotyledons.

Ascorbic acid is not generally regarded as an accessory growth factor for sterile cultures of tissues and roots, and only a few researches have been made in this field. Bonner and Axtman (4) found that the growth of pea embryos was favoured by 4 accessory substances including ascorbic acid. In a later paper (5) Bonner and Bonner showed that if another variety of pea was cultivated, an addition of ascorbic acid was ineffective, for this pea could satisfy its own requirements of that substance by synthesis. Thus this ability can vary, and it can be presumed that different results would be obtained by studying different plants. Bonner has autoclaved his media and by this process the acid must be oxidized (cf. Alg  us 1). The results of Bonner thus seem to be somewhat doubtful. An experiment with sterile-filtered media has been performed with the ascorbic acid concentration varying from 0.1 to 10,000 mg. per litre, though the acid is oxidized also by this treatment. As the substance is a rather strong acid the pH values in the solutions with more than 100 mg. per litre decreased from 5.8 to 3.6. The pH-values were not corrected by buffer solutions. Naturally no or very poor growth took place in these cultures, but there were no positive growth

effects in smaller concentrations either. If the acid had not already been destroyed before the inoculation, it could be stated that the lacking growth substance in isolated roots was not ascorbic acid.

c. Effects of plant extracts

It seems probable that if there are special substances promoting the meristematic cell divisions in the roots of monocotyledons these are to be found partly in germinating endosperm and partly in leaves, and they should to be isolated from these organs. Some preliminary experiments were made by the author with autoclaved press juice from germinating seeds and leaves from both barley and oats. The results were very disappointing, however, as the roots died just after inoculation. Some toxic compounds seemed to be formed by the process of autoclaving. Even if the hypothetical growth substances remain intact in this procedure, their effects can be masked by newly formed products (cf. Solomon's results with malt, 23). It is well-known that hitherto it has been impossible to cultivate plant tissues or organs in plant extracts with the exception of the experiments with coconut milk by van Overbeek (14) and Duhamet (8). Thus all preparations of tissue juices, xylem sap, phloem exudate and liquid endosperm have a toxic effect (White 25). This fact is in contradiction to the technique for cultivating animal tissues. The failure must probably be ascribed in part to erroneous methods. Thus the extracts generally have been autoclaved, except by McClary (13). Coconut milk has not yet been tested on these roots but experiments have been carried out with extracts of barley, oats, wheat, peas and most recently with a special type of extract, namely bleeding sap of *Carpinus betulus*. It has been very difficult to duplicate the results with new extracts.

In the experiments given below extracts of endosperm, leaves, and roots were prepared separately. The methods have not been exactly the same, but the variations are described. As media with plant extracts contain proteins, the procedure of sterile-filtration was often very time-consuming.

Experiment 1. The endosperm extract was made as follows. 200 seeds of barley and oats were germinated in Petri dishes for 4 days. Then the endosperms were removed and ground together in a mortar with water at room temperature. The extract was first filtered through paper and then through a glass filter funnel in order to remove the starch grains. The filtrates were then diluted to 200 ml. and added to the concentrated basic medium. Then this mixture was diluted to such a volume that the concentration of the components were the same as in normal nutrient solution (Table 10).

The effect of the endosperm extract in this experiment is rather insignificant. The material, however, was heterogenous and in one flask with 20

Table 10. *The effects of endosperm extracts on the growth of isolated roots of barley and oats.*

Sterilization method		Without extract		With extract	
		Autoclav. 20 min. at 2 atm.	Sterile filtration	Sterile filtration 1 0/o extract	Sterile filtration 20 0/o extract
Root length mm.	Barley	36	44	43	36
	Oats	33	38	37	38

Table 11. *The effects of different types of plant extracts on growth of isolated roots of barley and oats.*

Type and conc. of extracts		0	Endosperm extract		Root extract		Leave extract	
			25/150 ml.	75/150 ml.	25/150 ml.	75/150 ml.	25/150 ml.	75/150 ml.
Root length mm.	Barley ...	29	39	10	48	47	25	13
	Oats	38	28	10	23	24	32	17

per cent of the extract the barley roots had elongated extraordinarily, and, in spite of this, they had a large meristem residue. This result, however, could not be repeated. The cell divisions did not seem to be otherwise influenced.

Experiment 2. 10 gm. of seeds of barley and oats were germinated for 4 days. Coleoptiles, roots and endosperms were separated and ground separately in a mortar. The extracts were centrifuged and the plant material washed twice. The added portions were diluted to 200 ml. and the solutions used for preparing the nutrient media as mentioned above (Table 11).

Increased concentration of both endosperm extract and leaf extract inhibited root growth of barley and oats so that the roots died. Root extract had a positive effect on the elongation of barley roots, but a slight inhibition on oat roots. An increased concentration of root extract seemed to have no influence on barley roots. The oat roots were also unaffected by such an increase, but they did not like root extract at all, even in a low concentration.

Experiment 3. This was a repetition of experiment 2 with some modifications. 20 gm. of seeds of each species were germinated, and the separated parts were ground. The extracts were centrifuged and diluted to 200 ml. A portion of 100 ml. was extracted twice with ether in order to remove substances of auxin character. The water phase was freed from ether by aeration and the ether extract was evaporated to dryness and the residue dissolved in water (Table 12).

In conformity with experiment 2. leaf extract inhibited the growth of both kinds of roots when added in high concentrations. The endosperm frac-

Table 12. *The effects of different types of plant extracts on growth of isolated roots of barley and oats. a) Ether-treated extracts. b) Ether extract.*

a)

Type and conc. of extracts		0	Endosperm extract		Root extract		Leaf extract	
			10/150 ml.	50/150 ml.	10/150 ml.	50/150 ml.	10/150 ml.	50/150 ml.
Root length mm.	Barley ...	39	60	67	45	32	36	10
	Oats	41	36	28	30	33	22	10

b)

Extract		Endosperm	Root	Leaf
Root length mm.	Barley	52	33	28
	Oats	—	21	—

tion had a positive effect on the elongation of barley roots, as the roots became long and thin and all meristem cells had disappeared after 10 days of cultivation. The oat roots were not affected. The results, however, indicated that there is an active cell-elongation promoting factor in endosperm extract of barley, which is water-soluble and thus not identical with the general auxins. Probably it is a decomposition product of proteins.

Experiment 4. In experiments 1, 2, and 3 only 4-day-old plants were used. In this experiment extracts were prepared from 140 plants of each species, which had been grown for 12 days in light in a complete nutrient solution. Juices of leaves and roots were pressed separately and were diluted to 50 ml. In this experiment each extract was given to both species, but the table is incomplete as all cultures with barley root extract become infected, probably due to an accident in the sterile filtration (Table 13).

The extracts of barley leaves showed a slight positive effect on barley roots if the concentration was increased, but killed the oats roots completely. In extract of oat leaves in high concentration both types of roots were killed. Oat root extract promoted the growth of oat roots but not barley roots. These results indicate, that the growth of these types of roots is regulated by specific substances, although it was only the elongation process which was influenced. In the meristem no differences could be detected. Thus the results do not seem to be caused by meristem-activating substances.

Experiment 5. As the roots of dicotyledons generally grow when they are isolated, germinating seeds of such plants perhaps contain cell division-inducing compounds. In order to study, whether they had beneficial effects on roots of monocotyledons, extracts were prepared of pea, »Svalöfs Torsdagsärt». 25 gm. of peas were soaked for 12 hours in aerated water, ground in a mortar and then press juice was made. It was diluted to 200 ml. and divided into 2 portions, one of which was autoclaved for 60 min. at 1 atm.

Table 13. *Root lengths in mm. of barley and oats cultivated in nutrient media with extracts of 16-day-old plants.*

Extract	Leaves of barley		Leaves of oats		Roots of oats	
	5/250 ml.	45/250 ml.	5/250 ml.	45/250 ml.	5/250 ml.	45/250 ml.
Barley	21	38	34	15 (dead)	32	22
Oats	10 (dead)	10 (dead)	30	10 (dead)	32	42

Table 14. *Root lengths in mm. of barley and oats cultivated in nutrient media with pea extract.*

Type of extract	0	Not autoclaved			Autoclaved		
		1/200 ml.	10/200 ml.	50/200 ml.	1/200 ml.	10/200 ml.	50/200 ml.
Barley	29	38	32	10 (dead)	—	50	20
Oats	25	25	20	10 (dead)	—	21	18

The prepared media were very difficult to filtrate through a membrane filter (Table 14).

As is seen from the table, there were no positive results. It is true that some barley roots had elongated markedly, but they were tapering and had lost their meristems.

Often in the literature plant extract has been added to culture media in order to improve growth. Robbins and Maneval (20) discussed some experiments with corn roots, to which extracts of corn grains, corn plant, corn endosperm, Canada field pea, and clover were added, but in no cases was there any striking improvement in growth. The solutions were autoclaved intermittently. Robbins and White (21) had tried milk of immature corn grains, diffusate from germinated grains, water extracts of roots, germinating grains etc. Their results were also mainly negative and agreed with those in this paper. Loo and Loo (11 and 12) have made some studies on the effects of leaf extracts on the growth of corn root tips. They did not try to have unlimited growth but only studied the effects of different leaf extracts on root length and laterals in order to verify Went's theory on rhizocalin. The authors assumed, however, that there is growth-promoting material in leaf extracts which promotes growth by increasing cell divisions and cell elongation. Their roots, however, looked very malformed and seem not to be normal in any case. Extracts of such a type have little value in the present investigation.

The bleeding sap of trees in spring contains sugars, minerals, growth substances etc. and it could be assumed that the latter would have a bene-

Table 15. *The inhibitory effect of bleeding sap of Carpinus betulus on root growth of barley and oats.*

Conc. of bleeding sap		5/250 ml.	40/250 ml.	80/250 ml.	pure sap
Root length mm.	Barley	35	25	18	17
	Oats	29	21	19	16

ficial effect on root growth. For these experiments bleeding sap of *Carpinus betulus* was available. Its glucose concentration was determined to 0.1 mol per litre. In the first experiment the sap was added to the basic medium. In the second it was diluted with redistilled water, but here the growth was very poor and most of the roots died. The results of the former experiment are given in Table 15.

Thus the addition of bleeding sap only had an inhibitory effect on the root growth, probably due to auxins. Even if these substances were removed by ether extraction, no positive increase in growth was obtained.

d. Effects of organic nitrogen compounds

As has been related earlier (2) nitrate is the nitrogen source in the normal basic medium. Nitrate is absorbed but its assimilation has not yet been studied thoroughly on this material. Ammonium is a bad poor source of nitrogen. Furthermore, some amino acids have been tested previously, namely, glycine, aspartic acid and cysteine hydrochloride, all without effects on growth and meristematic activity. White (24) could replace a yeast extract fraction made with 85 per cent alcohol with a mixture of 9 amino acids, so the amino acids are beneficial to the tomato roots. According to Virtanen (cf. Rautanen 18) amino dicarboxylic acids, which are generally regarded as intermediary products in nitrate assimilation, cannot function as nitrogen sources for grass, but asparagin is excellent. His studies, however, are made on intact sterile plants. As already mentioned aspartic acid was ineffective for roots of barley and oats and the same fact was found when glutamic acid and also asparagine were tested. Naturally this finding does not prove that asparagine is a poor source of nitrogen, for probably other factors are limiting the growth. In order to elucidate this problem further some studies were made on more complex nitrogen compounds such

Table 16. *Effects of peptone on root growth of barley and oats.*

Concentration mg/l.		0	10	100	1.000	10.000
Root length mm.	Barley	36	46	43	39	10
	Oats	30	30	26	15	10



Fig. 1. *Typical excised root tips of barley cultivated for 10 days in darkness at 20° C. Enl. 49 X. a. In normal basic media. Most meristem cells have elongated, b. In basic media supplied with 1000 mg. peptone per litre. Note the relatively large meristem residue and greater root diameter.*

as gelatine, peptone (both preparations Coleman and Bell) and Casamino Acids (vitamin-free hydrolyzed casein, Difco).

Gelatine was given in 1 gm. per litre, thus in liquid solution, but no special effects could be detected.

Robbins (19) states that peptone was beneficial to the growth of isolated corn roots, but indefinite growth was impossible to obtain. 0.04 per cent of peptone, however, increased the meristematic activity so that the roots could be subcultured 4 to 6 times in comparison with 3 times normally before the cessation of growth.

The barley roots in 1000 mg. peptone per litre were very interesting. They had relatively long meristem residues (Fig. 1), great root diameters and short, thick laterals. Thus the root type was not the tapering one, but more normal and healthy. The oat roots, however, did not seem to be affected as usual. After 10 days' culturing in the media, some roots were transferred to new media of the same composition, but then the growth stopped gradually, even in the case of the barley roots. The meristem residues were very small. Thus the growth could not be maintained due to deficient meristematic activity. The longest barley roots after 20 days of cultivation were about 90 mm. and more tapering than after 10 days.

In order to concentrate and identify the active factors in peptone some extractions were performed with ether and alcohol (96 per cent and absolute

Table 17. *Effects of different types of peptone extracts of root growth of barley and oats.*

Type and conc. of extract		0	96 % alcohol in Soxhlet apparatus			Ether extraction after alcohol treatment	Direct ether extraction
			1/200 ml.	2/200 ml.	10/200 ml.		
Root length mm.	Barley ...	38	43	37	36	35	27
	Oats	31	28	26	20	22	19

Type and conc. of extract		Abs. alcohol by shaking			Ether extr. after alcohol treatment	Residue after extraction in alcohol and ether	
		1/200 ml.	2/200 ml.	10/200 ml.		1/200 ml.	2/200 ml.
Root length mm.	Barley ...	45	54	45	39	43	55
	Oats	34	32	24	26	25	17

Table 18. *Effects of Casamino Acids on root growth of barley and oats.*

Concentration mg/l.		0	10	100	1.000	10.000
Root length mm.	Barley	33	32	49	43	25
	Oats	26	31	33	39	19

alcohol). 10 gm. of peptone were extracted in a Soxhlet apparatus or by shaking in a separatory funnel. The extracts were evaporated and the residues dissolved in water to 100 ml. Thus 10 ml. extract were equivalent to 1 gm. peptone (Table 17).

In general the barley roots were more affected than the oat roots, the growth of which in most cases was inhibited, except in extracts in absolute alcohol. In summarizing these results it can only be stated that peptone had a beneficial effect on meristems of barley roots. The roots thicker and not so fragile as usual, indicating a better development of the stele. Further studies are in progress.

The preparation Casamino Acids also gave roots an appearance which diverged from the usual one obtained. They were relatively thick with short laterals. No meristem effects could be observed (Table 18).

Discussion

In the survey of cultivations of grass roots given by Bonner and Bonner (6) it is striking, that all experiments of culturing such roots have been negative, a fact which is also applicable to a certain extent to the culture

of tree roots (exceptions are *Acacia melanoxylon* and *Pinus silvestris*). Even if a researcher has announced successful results, e.g., McClary (13) with corn roots, other people have not succeeded in repeating them (6). Burström (private communication) has once cultivated excised roots of wheat with success for one month in a medium supplied with a yeast extract named Cenovis, not available now. The roots grew at a constant rate of about 10 mm. a day. He made no subcultures, however, but the fact indicates, that the problem is not insoluble.

McClary (13) has discussed the reasons for the cessation of the growth of roots of monocotyledons after isolation, and proposed such factors as the excision of the vessels and the external supply of sugars. This is also the case in cultures of dicotyledons. He further discovered that growth was very poor in aqueous solutions, but it improved in solid agar media. In such media he obtained his successful results with 18 subcultures without diminishing vitality. These experiments indicated that there could be difficulties in the gas exchange of roots, but both aeration and agar media have not had any positive effect on barley and oat roots. The intercellulars always contain gas.

When comparing the growth of roots of intact plants of, for example, wheat, tomato and pea, it is reported (Hayward 10), that the seminal roots of wheat can grow to 8 to 12 inches and the decay and the adventitious roots take over their functions. On the other hand tomato roots grow 1 inch a day up to 2 feet, and the primary root of pea can also elongate to 4 feet. Thus growth differences are already present before the isolation of the root tips, which then increase to a still greater extent. No culture experiments of the tips of the adventitious roots have been performed, but perhaps these will behave in a different way, for it seems as they are more vigorous already when attached to the plant. It may be assumed that these intrinsic differences in growth have their origin in specific growth substances.

It is a curious fact that embryos of grasses without endosperm cultivated in sterile sugar solutions show the same lacking meristematic activity in the roots. The following experiment was performed in order to investigate whether any growth-promoting substances are synthesized by the bacteria in the nutrient media. Intact plants of barley and oats were planted in a nutrient solution and after 5 days glucose solutions were added. After another 5 days the media were opaque due to bacterial activity. The solutions were autoclaved and given in varied portions to normal basic medium, but without any promoting effects.

The results described in this paper have shown that in the autoclaving process no toxic compounds are formed which are responsible for the lack of growth and, furthermore, such accessory growth factors as indole

acetic acid and thiamin are not destroyed. As is evident from the experiment with sodium arsenate, the inhibited growth is not caused by a surplus of auxins. It has been impossible to find a meristem-promoting substance in different types of plant extracts. Its effects must be masked, or it is very easily destroyed, when the plantcells are killed. Peptone is the only substance hitherto that has given any result, at least temporarily. Its effects are to be found in the meristems and in a good appearance of the roots, not specially in root lengths. It is beneficial, however, only to barley and not to oats. This fact indicates that the growth of different species of grasses is not promoted by identical substances. Moreover, the peptone factor is not adequate for the meristem growth, but it must be supplemented by others.

The fact that the lacking growth substance cannot be obtained from any other parts of the same plant shows that it is never formed in abundance but apparently always in suboptimal quantities, immediately consumed up by the root meristems. As already mentioned, this might be connected with the normally limited elongation of the roots of monocotyledons, so that these two phenomena might illustrate two sides of the same metabolic property, peculiar to the monocotyledons.

Summary

Earlier studies on the growth requirements of isolated roots of barley and oats have been continued by using the culture methods already described. The investigation has included 3 problems.

- a. Effects of the method of sterilization. A comparison of autoclaving and sterile filtration in membrane filters after Zsigmondy on the following substances: glucose, sucrose, fructose and the accessory growth factors indole acetic acid, thiamin, and ascorbic acid.
- b. Effects of extracts of endosperm, roots and leaves of barley and oats, endosperm of pea, and bleeding sap of *Carpinus*.
- c. Effects of some nitrogen compounds, namely glutamic acid, asparagine, gelatine, peptone, and Casamino Acids (hydrolyzed casein).

In most cases no promoting effects on the meristematic activity could be observed, but the added substances generally caused variation in root elongation. However, peptone in a concentration of 1 gm. per litre was beneficial to barley roots, which, when examined after 10 days' cultivation, had extraordinarily long meristem residues, and after 20 days some non-elongated cells also remained. The roots were thick and not very tapering.

The other results indicated that the factors under investigation could not be responsible for the inhibited growth of roots of monocotyledons after isolation. The results with peptone indicate, however, that there is some definite growth factor lacking, and that the failure does not depend upon the internal constitution of the roots. Further it proved impossible to isolate the hypothetical growth factor from extracts of endosperm, roots and leaves of corresponding plants. The results with extracts from endosperm, however, indicated the presence of a water-soluble elongation factor, not of auxin character.

Further studies are in progress.

References

1. Algéus, S.: Untersuchungen über die Ernährungsphysiologie der Chlorophyceen. — Bot. Not. 1946: 129. 1946.
2. Almestrand, A.: Studies on the growth of isolated roots of barley and oats. — Physiol. Plant. 2: 372. 1949.
3. Bonner, J.: Arsenate as a selective inhibitor of growth substance action. — Plant. Phys. 25: 181. 1950.
4. — and Axtman, G.: The growth of plant embryos in vitro. Preliminary experiments on the rôle of accessory substances. — Proc. Nat. Acad. Sci., Wash. 23: 453. 1937.
5. — and Bonner, D.: Ascorbic acid and the growth of plant embryos. — Ibid. 24: 70. 1938.
6. — and Bonner, H.: The B vitamins as plant hormones. — Vitamins and Hormons. VI: 225. New York. 1948.
7. Burström, H.: Studies on the carbohydrate nutrition of roots. — Ann. Agr. Coll. Sweden. 9: 264. 1941.
8. Duhamet, L.: Action du lait de coco sur la croissance des tissus du tubercule de Topinambour cultivés in vitro. — C. r. acad. sci., Paris. 229: 1353. 1949.
9. Fries, N.: Über die Bedeutung von Wuchsstoffen für das Wachstum verschiedener Pilze. — Symb. Bot. Ups. III: 2. 1938.
10. Hayward, H.: The structure of economic plants. — New York. 1938.
11. Loo, T. L. and Loo, S. W.: Studies on the culture of isolated root tips under sterile conditions. I. The effect of leaf extract on the growth of root tips. — Sci. rep. nat. cent. univ. Nanking, Ser. B. Biol. 2: 51. 1935.
12. — and — Studies on the culture of isolated root tips under sterile conditions. II. Further experiments on the effects of leaf extract on the growth of root tips. — Chinese Jour. Exp. Biol. 1: 189. 1936.
13. McClary, J. E.: Synthesis of thiamin by excised roots of maize. — Proc. Nat. Acad. Sci., Wash. 26: 581. 1940.
14. van Overbeek, J., Siu, R., and Haagen-Smit, A. J.: Factors affecting the growth of *Datura* embryos in vitro. — Am. J. Bot. 31: 219. 1944.
15. Padmasini, K.: Antiseptic culture of ragi (*Eleusine corocana*) seedlings and their response to vitamins. — Current sci. 17: 227. 1947.
16. Philipson, T.: Über die quantitative Bestimmung reduzierender Zucker. — Ark. Kemi. 16 A. No 22. 1943.
17. Prát, S.: The toxicity of tissue juices for cells of the tissue. — Am. J. Bot. 14: 120. 1927.

18. Rautanen, N.: On the synthesis of the first amino acids in green plants. — Ann. Acad. Scient. Fenn. Ser. A. II. Chemica 33. 1948.
19. Robbins, W.: Effect of autolized yeast and peptone on growth of excised corn root tips in the dark. — Bot. Gaz. 74: 59. 1922.
20. — and Maneval, W.: Further experiments on growth of excised root tips under sterile conditions. — Ibid. 76: 274. 1923.
21. — and White, V.: Effect of extracts from the corn plant on growth of excised root tips. — Ibid. 98: 520. 1937.
22. Smith, M. L.: The effect of heat on sugar solution used for culture media. — Biochem. Jour. 26: 1467. 1932.
23. Solomon, B.: Inhibiting effect of autoclaved malt preventing the in vitro growth of *Datura* embryos. — Am. J. Bot. 37: 1. 1950.
24. White, Ph. R.: Amino acids in the nutrition of excised tomato roots. — Plant. Phys. 12: 793. 1937.
25. — A handbook of plant tissue culture. — Lancaster 1943.

The Utilization of Aspartic Acid, Succinamide and Asparagine by *Scenedesmus obliquus*

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Since early times asparagine has been considered to occupy a very central position in the nitrogen nutrition of plants. Its accumulation at the germination of the higher plants in darkness and its disappearance in light were regarded from the outset as a sign that the substance had the function of a nitrogen depot in which nitrogen from the reserve protein could be temporarily stored for later utilization in protein synthesis when a sufficient supply of carbohydrate was present. Aspartic acid has also been ascribed the function of binding ammonia and thereby protecting the cell from NH_3 poisoning. Later investigations of the roll played by asparagine have confirmed in essentials the view of asparagine as an important intermediary link in the nitrogen nutrition of plants, at the same time as they have naturally given us a deeper insight into the complicated processes of reaction. Thus, the carbon chain of asparagine is derived from the oxaloacetic acid formed in the acid cycle of respiration, the nitrogen from hydrolysis of the proteins. From the two nitrogen groups of asparagine the amido-nitrogen, which is not capable of transamination (Virtanen and Laine 13), must be split off as ammonia, while the amino-nitrogen can be converted by transamination into alanine and glutamic acid, possibly also other amino acids necessary in protein synthesis.

The nitrogen sources of the higher plants are as a rule nitrate or ammonium salts. Organic compounds may also be utilized, however. According to Rautanen (12), aspartic acid is absorbed by peas without the quantity of asparagine being increased. He takes this as an indication that aspartic acid is a very active compound. At all amide formation, irrespective of the

nature of the N source, glutamine was formed in the first place, later asparagine. Amide formation proceeded without free ammonia being demonstrable as an intermediary compound. The value of aspartic acid and asparagine as source of nitrogen for the higher plants depends on a long series of factors such as transamination, deamination and the plant's supply of enzymes that catalyse these reactions. — It is a well-known fact that lower heterotrophic organisms, fungi and bacteria, can use manifold organic compounds to supply their nitrogen requirements. α -amino acids are usually broken down during deamination, the ammonia formed being regarded as the primary source of nitrogen in the protein synthesis. In acid solution the deamination is replaced by a decarboxylation with formation of organic amines (Gale 7), some of which latter can be utilized. Transamination with production of aspartic acid and alanine from glutamine is also known (Lichstein and Cohen 10). The mechanism by which the amino acids penetrate the cell was studied by Gale (8). He was able to show that *Streptococcus faecalis* took up lysine and glutamic acid, the former by ordinary diffusion, the latter with utilization of energy formed by glycolysis.

An intermediate position between the higher autotrophic plants and the heterotrophic micro-organisms can be said to be occupied by certain planktonic green algae. On one hand, these have a purely autotrophic mode of nutrition, on the other, when there is a supply of organic substances, they can assimilate these. That aspartic acid and asparagine can then be utilized as N source, is a well-known fact, though reports vary as to their qualities in this respect. They are often described as extraordinarily good nitrogen sources. Kossowitsch (9) and Artari (5), however, state that asparagine is inferior to several other nitrogenous compounds such as glycocoll and alanine. Algéus (1) came to a similar result. In view of the key position asparagine generally is considered to occupy in nitrogen metabolism this seems surprising. The mechanism underlying the penetrance of these compounds into the algal cell is still completely unknown, as are also the changes they undergo during assimilation. The purpose of the present work is to investigate the nutritional value of aspartic acid and asparagine for a planktonic green alga in pure culture by means of a quantitative study of nitrogen assimilation, ammonia formation and growth while comparing the results thus obtained with those earlier found by the author in his studies of other amino acids.

Methods

The methods employed for these investigations have been previously described in detail (Algéus 1, 2). Over and beyond this description the following points may be added. The stock solution had the following composition: MgSO_4 , $7\text{H}_2\text{O}$ 0.02,

CaSO_4 , $2\text{H}_2\text{O}$ 0.02, K_2HPO_4 0.02, Fe-citrate 0.002 g per litre solution. The organic nitrogen-containing substances, dl-aspartic acid, dl-asparagine as well as succinamide are used in a concentration corresponding to 10 mg N per culture. Nitrogen is then present in excess. The pH was adjusted to about 7.0 with NaOH. In experiment 2 reported below it was found necessary to have recourse to a phosphate buffer in the concentration 1/600 M on account of pH shifts at the sterilization. As the organic substances are apt to be hydrolysed on being heated to 120°C , recourse had to be had to fractional sterilization with heating to 100°C . The pH and absence of ammonium ions were checked after equilibrium with the air gases had appeared. Determinations of growth and pH as well as of assimilated nitrogen and ammonia were as a rule made every three days. All experiments were carried out in light with atmospheric carbon dioxide as a source of carbon. Experimental organism: *Scenedesmus obliquus*.

Experimental Results

Experiment 1. Aspartic acid. The experimental results are shown in Fig. 1. Cell division took place at an almost constant rate during the course of eighty days, after which the experiment was discontinued. The rate of cell division, however, was low and the final cell count in the solution was approximately 6,000 per mm^3 . Compared with the growth curves obtained with other amino acids (Alg  us 2, 3, 4) the aspartic acid curve is remarkable because of the constant growth throughout the entire experimental period. It will be further discussed below. Fig. 1 also shows the nitrogen content of the cultures at different points of time. The nitrogen assimilation also seems to take place at a constant rate without any significant retardation. The nitrogen content is low, approximately 0.2 mg per culture. Ammonia could not be demonstrated in the culture medium except on one single occasion (after 36 days), when traces could be detected. The pH, which in the beginning was 7.0, showed only insignificant variations of ± 0.1 . The cultures exhibited a normal appearance and had a beautiful green colour. To begin with the nitrogen content of the individual cell (Fig. 2) was rather high, approximately $20 \cdot 10^{-10}$ mg per cell or even higher (some points are not included in the figure), but it fell during the first week to between 6 and $10 \cdot 10^{-10}$ mg. The final values also lie in this range and must be considered low.

The result of the experiment may be summarized as follows: *Aspartic acid can be utilized as a source of nitrogen by Scenedesmus obliquus. The cells exhibit a normal appearance but the rates of cell division and nitrogen assimilation are low. Since no secondary retardations in growth and assimilation occur, the experiment indicates that the amino-N is utilized with some difficulty, thereby keeping the cell number and protein content at a low level. In agreement with these findings is the fact that no ammonia is found in the nutrient solution.*

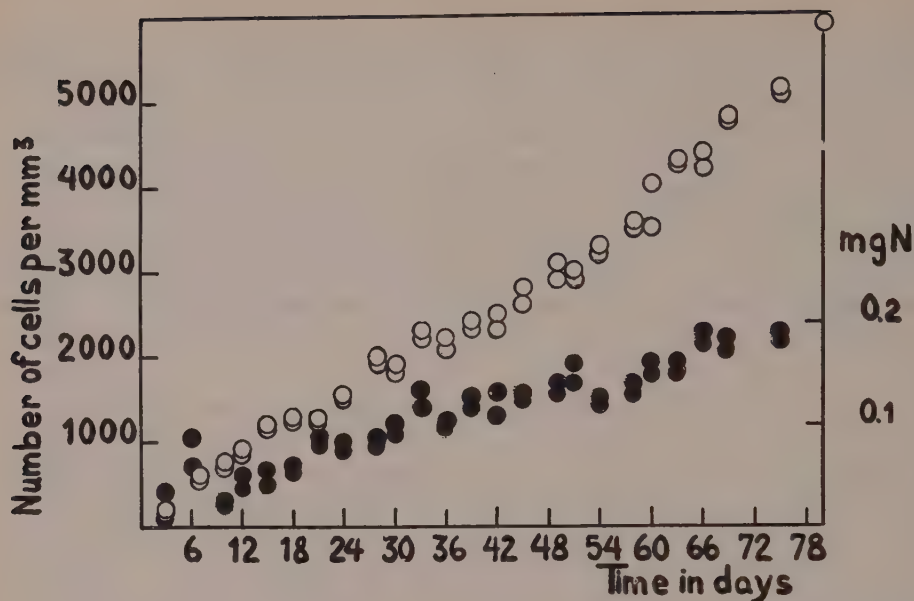


Fig. 1. Growth and nitrogen assimilation in cultures with aspartic acid. The abscissae give the time in days, the ordinates the number of cells per mm³ (○) and the assimilation in mg N per culture (●).

Experiment 2. Succinamide. Before experimenting with asparagine it seemed expedient to test the power of the algae to utilize amido-N in succinamide (the half amide, which would have been still more suitable for the purpose, could unfortunately not be obtained). The results of the experiment are shown in Fig. 3. The maximal cell count, approximately 6,000 cells per mm³, was already reached after eighteen days, when a retardation in growth took place. N-assimilation ceased about the same time as the cell division and the N-content of the culture was comparatively low, approximately 0.3 g. Ammonia was present throughout the entire experiment, although the concentration was highest in the beginning (about 0.1 mg per culture). The pH showed small variations between 7.0 and 7.2. The cells had a normal appearance and were beautifully green. At first the nitrogen content of the individual cell (Fig. 2) was high (some values are not included in the figure), but it decreased during the first few weeks to approximately $10 \cdot 10^{-10}$ mg. After forty days the experiment was discontinued, as the cell number and nitrogen content were constant.

Although the final results in the experiment with succinamide strongly resemble those obtained with aspartic acid, the course of the experiment

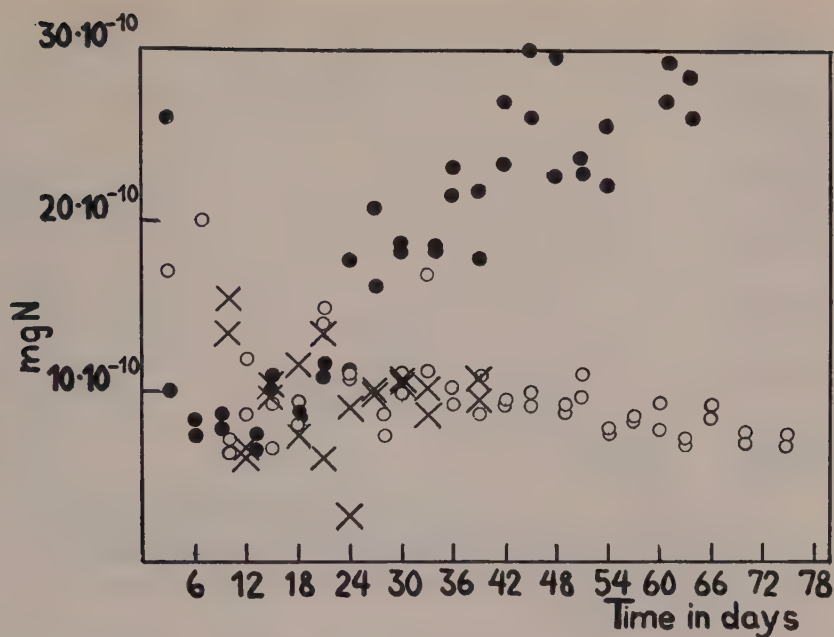


Fig. 2. Nitrogen content of the individual cell. The abscissae indicate the time in days, the ordinates the N-content per cell in mg. Cultures with aspartic acid (O), with succinamide (X) and with asparagine (●).

was entirely different. The cell count, which was 6,000 per mm^3 in both cases, was reached with aspartic acid after approximately eighty days, while with succinamide this number was attained after approximately eighteen days. In the former case the growth took place at an almost constant rate without any retardation, while in the latter there was no cell division after the eighteenth day. What has been said here of the growth is also largely applicable to the N-assimilation. In the aspartic acid experiment it took place at an almost constant rate, while with succinamide the rate was higher but it was soon completely inhibited. Slightly more nitrogen was assimilated in the latter case. Since these two experiments involve amino-N and amido-N respectively, the mechanism of N-assimilation must be quite different. In the case of aspartic acid it may be a question of a transamination or an oxidative deamination, while with succinamide it is presumably a hydrolytic deamidation. In the former it seems as if the deamination or transamination were the limiting factor. During the assimilation of the amino acid no inhibitory substances appear and the growth proceeds continuously. *In the experiment with succinamide, nitrogen is released in excess during the*

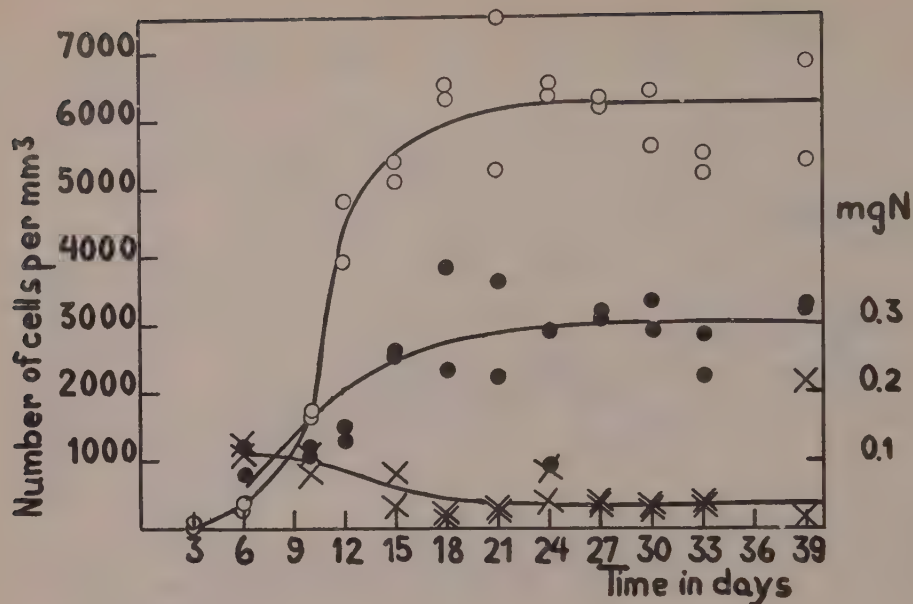


Fig. 3. Growth, nitrogen assimilation, and ammonia production in cultures with succinamide. The abscissae give the time in days, the ordinates the number of cells per mm³ (○—○), as well as the nitrogen assimilation (●—●) and the ammonia production (×—×) in mg N per culture.

deamidation and is found in the nutrient solution in the form of ammonia. In this instance the nitrogen does not limit either the protein synthesis or the growth. The rates of assimilation and of cell division are high. The fact that they cease so comparatively soon and that the N-content per cell, even in this case, is low, must be related to the formation of inhibitory substances during the deamidation.

Experiment 3. Asparagine. Asparagine combines the properties of aspartic acid and succinamide in that it contains both amino-N and amido-N. A summary of the results of the experiment is submitted in Fig. 4. The rate of growth is greater than with aspartic acid but less than with succinamide, with a retardation after approximately fifty days. The final number of cells was higher than in the two preceding experiments, amounting to 10,000 per mm³. This is due to the greater rate of cell division and to the later-occurring retardation. The N-assimilation also proceeded, to about the fifteenth day. At that time the N-content of the culture was approximately 1.5 mg and thus considerably exceeded that found in the two preceding experiments. Ammonia was present throughout the greater part of the experiment, with the excep-

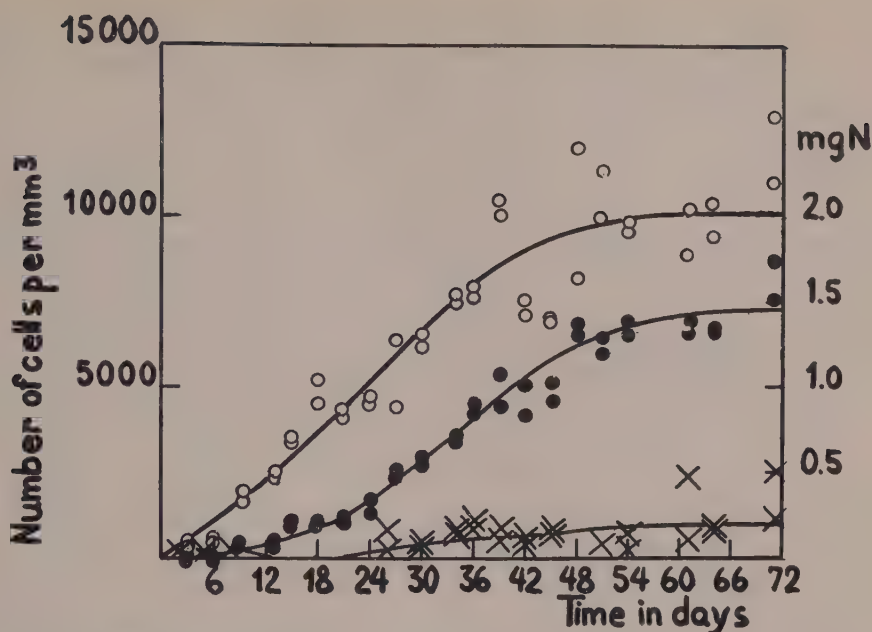


Fig. 4. *Growth, nitrogen assimilation and ammonia production in cultures with asparagine.* The abscissae give the time in days, the ordinates the number of cells per mm³ (○—○), as well as the nitrogen assimilation (●—●) and the ammonia production (×—×) in mg N per culture.

tion of a period between the tenth and twenty-fifth day. The amounts were generally small, approximately 0.2 mg per culture. The cultures were dark green and the final nitrogen content of the cells was high, $30 \cdot 10^{-10}$ mg per cell (Fig. 2).

It may be of interest to compare the average amount of nitrogen split off (assimilated N + ammonia-N) by the individual cell in the different experiments. The calculations in mg N per cell per day are shown in Table 1. They were made in accordance with previously mentioned principles (Algéus 2) and cover the time from the beginning of the experiments to the cessation of the nitrogen assimilation and ammonia production. *In accordance with the view that amido-N and amino-N are split off by entirely different mechanisms the rates of N-utilization show wide variations. With succinamide it is thus sixteen times greater than with aspartic acid. Asparagine gives a value which is closest to that of succinamide, thus indicating that the amido-N is the first to be utilized. This is supported by chemical analyses.* Table 2 shows the amount of assimilated nitrogen and ammonia nitrogen in

Table 1. *The average amount of nitrogen split off (assimilated N+ammonia-N) by the individual cell in mg per cell per day.*

Aspartic acid	Succinamide	Asparagine
$0.2 \cdot 10^{-12}$	$3.2 \cdot 10^{-12}$	$1.6 \cdot 10^{-12}$

Table 2. *The protein and ammonia content of the cultures and the amounts of amido- and amino-N utilized.*

Protein-N, mg	2.25	1.83
Ammonia-N, mg	0.66	1.18
Total N, mg	2.91	3.01
Utilized amido-N, mg	2.88 ₁	2.89 ₁
Utilized amino-N, mg	0.03	0.12
Total N, mg	2.91	3.01

¹ Calculated by difference.

two cultures about four months old as well as the amount of utilized amido-N and amino-N. It is evident that the alga consumes practically solely the former, the amount of amino-N utilized being insignificant or none whatsoever. — For a description of the methods used for determining the amido-N reference may be made to Winterstein (14). The amino nitrogen has been calculated by difference between utilized total-N and utilized amido-N.

Discussion

The experiments show that the amino-N in aspartic acid can be utilized as source of nitrogen by *Scenedesmus obliquus*, even if with some difficulty. Everything would seem to indicate that deamination or transamination processes have a limiting action on protein synthesis and growth. No injurious metabolic products are formed at the utilization, for the rates of growth and assimilation still remain constant after three months. The amido-nitrogen in the succinamide is readily utilized with liberation of ammonia to the solution. However, a retardation of growth and protein synthesis probably caused by some intermediary substance formed at the deamidation sets in at an early stage. The nature of the inhibitory substance is at present unknown. Should it be a question of a hydrolytic deamidation, however, a feasible assumption is that succinic acid or one of its decomposition products is responsible. If the alga is offered amino- as well as amido-nitrogen

Table 3. *A comparison between glycocoll, alanine, aspartic acid, succinamide, and asparagine as a source of nitrogen for Scenedesmus obliquus.*

Property studied	Glycocoll	Alanine	Aspartic acid	Succinamide	Asparagine
Cells per mm ³	20,000	10,000	6,000	6,000	10,000
Assimilated N; mg per culture	1.5	1.7	0.2	0.3	1.5
N-content per cell; mg	$15 \cdot 10^{-10}$	$34 \cdot 10^{-10}$	$10 \cdot 10^{-10}$	$10 \cdot 10^{-10}$	$30 \cdot 10^{-10}$
Rate of N-assimilation and ammonia production of the cell (average value); mg per cell per day.....	$3.7 \cdot 10^{-12}$	$1.1 \cdot 10^{-12}$	$0.2 \cdot 10^{-12}$	$3.2 \cdot 10^{-12}$	$1.6 \cdot 10^{-12}$
Ammonia-N; mg per culture	1.0	0.2	0	0.05	0.2
Final pH	8.2	7.5	7.0	7.0	7.0
Time for N-assimilation in days	30	> 60	> 80	18	50

in the form of asparagine, mainly the later is utilized. This is evident from the velocity at which the nitrogen is assimilated as well as from chemical analyses. The product developed at the deamidation of asparagine does not have a retardative action, and hence cell division and N-assimilation can proceed undisturbed for a longish time. It is probable that aspartic acid is formed and, according to experiment 1, this substance has no secondary effect. The retardation at last appearing in the asparagine experiment may possibly be caused by an auto-intoxication in the culture (Pratt 11).

It may be of interest to compare the nitrogen sources used here with those earlier studied (Alg  us 2, 4). Although the comparison is to some extent rendered difficult by secondary pH changes in the solutions, Table 3 nevertheless gives a good idea of the utility of the different N-compounds. *The table shows that glycocoll is consumed with the greatest rapidity. Close to it comes succinamide, though this gives quite a different final result as regards cell number, nitrogen content and ammonia content owing to the short time of assimilation, 18 days. Alanine and asparagine are comparable in several respects, though this cannot be associated with an agreement in the mechanism of the chemical reaction at their assimilation, since in one case it is the amino-nitrogen and in the other the amido-nitrogen which is utilized. Aspartic acid is inferior to the other compounds and, moreover, differs from them by giving a constant growth and a constant rate of nitrogen assimilation during the whole period of the experiment.*

That aspartic acid is the poorest of the nitrogen sources studied here and that asparagine cannot match, e.g., glycocoll may seem surprising in view of the central position usually ascribed to these substances in the nitrogen metabolism of the plants, a problem complex that has been touched upon

in the opening remarks of this paper. However, for many plants the key position earlier ascribed to aspartic acid and asparagine appears in reality to be held by glutamic acid and glutamine. The latter compound, for instance, appears to play a considerably more prominent part in the metabolism of barley than asparagine (Yemm 15). Here, the more stable asparagine is formed under conditions that can scarcely prevail during growth and development. The investigation into the amide formation in peas (Rautanen 12) points in the same direction. In a hydrolysate of *Chlorella* cells, Eny (6) was able to demonstrate the occurrence of glutamic acid as well as aspartic, the former in larger amounts than the latter. *It may be assumed that the key position in the nitrogen metabolism of Scenedesmus obliquus is not occupied by aspartic acid but by glutamic acid*, similar to what is the case in many higher plants. Whether it is the latter compound that constitutes the important connecting link between nitrogen and carbon-hydrate metabolism in algae can, however, only be decided by continued experiments.

Summary

1. Pure cultures of *Scenedesmus obliquus* were grown with aspartic acid, succinamide and asparagine as a source of nitrogen, and growth, nitrogen assimilation and ammonia production were determined every three days.

2. In the aspartic acid cultures cell division took place at a low but constant rate for three months. The N-assimilation was also low but constant. The nitrogen content of the individual cell amounted to approximately $6 \cdot 10^{-10}$ mg. Ammonia could not be demonstrated in the culture medium.

3. With succinamide the same final cell count was attained as with aspartic acid, viz. 6,000 per mm^3 , but growth was already completed after eighteen days. The N-assimilation ceased at the same time as the cell division, and the N-content of the individual cell was the same as in the preceding case. Ammonia was present in the nutrient solution throughout the entire experiment.

4. The rate of growth with asparagine was intermediate between the two foregoing. An inhibition in growth took place first after fifty days and therefore the final cell count was higher than in the two preceding experiments, 10,000 per mm^3 . The N-assimilation proceeded similarly to about the fiftieth day, when the N-content of the cells amounted to $30 \cdot 10^{-10}$ mg per cell.

5. In agreement with the idea that amido-N and amino-N are split off according to different mechanisms the rates of the N-utilization show great differences. With succinamide it is thus sixteen times greater than with aspartic acid. Asparagine resembles succinamide most closely, which indicates

that the amido-N is the first to be utilized. This had been confirmed by chemical analyses.

6. The experimental results have been discussed and the sources of nitrogen compared with one another. Comparisons have also been made with earlier studied amino acids, glycocoll and alanine.

7. The fact that aspartic acid is the poorest of the nitrogen sources has been discussed and the hypothesis set up that the key position in the nitrogen metabolism of *Scenedesmus obliquus* is not occupied by aspartic acid but by glutamic acid, similar to what is the case in many higher plants.

References

1. Algéus, S.: Untersuchungen über die Ernährungsphysiologie der Chlorophyceen. — Bot. Not. 1946:129. 1946.
2. — Glycocoll as a Source of Nitrogen for *Scenedesmus obliquus*. — Physiol. Plant. 1: 65. 1948.
3. — Deamination of Glycocoll by *Scenedesmus obliquus* in Intermittent Light. — Physiol. Plant. 2:145. 1949.
4. — Alanine as a Source of Nitrogen for Green Algae. — Physiol. Plant. 2:266. 1949.
5. Artari, A.: Der Einfluss der Konzentration der Nährlösung auf die Entwicklung einiger grüner Algen. II. — Jahrb. wiss. Bot. 43:177. 1906.
6. Eny, D. M.: Amino acids in healthy *Chlorella* cells. — J. Bact. 58:269. 1949.
7. Gale, E. F.: The Bacterial Amino-acid Decarboxylases. — Adv. Enzym. 6:136. 1946.
8. — The Assimilation of Amino-acids by Bacteria. I. — J. Gen. Microbiol. 1:53. 1947.
9. Kossowitsch, P.: Untersuchungen über die Frage, ob die Algen freien Stickstoff fixieren. — Bot. Ztg. 52:97. 1894.
10. Lichstein, H. C. and P. P. Cohen: Transamination in Bacteria. — J. Biol. Chem. 157:85. 1945.
11. Pratt, R.: Influence of the Size of the Inoculum on the Growth of *Chlorella vulgaris* in Freshly Prepared Culture Medium. — Am. J. Bot. 27:52. 1940.
12. Rautanen, N.: On the Synthesis of the First Amino Acids in Green Plants. — Ann. Acad. Sci. Fenn. Ser. A 2. Chem. 33:1. 1948.
13. Virtanen, A. I. und Laine, T.: Über die Umaminierung in grünen Pflanzen. — Bioch. Z. 308:213. 1941.
14. Winterstein, A.: Aminosäuren. — Handb. d. Pflanzenanalyse, herausg. v. G. Klein. 4/1, 3:1. Wien 1933.
15. Yemm, E. W.: Glutamine in the Metabolism of Barley Plants. — New Phytol. 48:315. 1949.

Cytophysiological Studies on *Micrasterias* II. The Cytoplasmic Framework and its Mutation

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Introduction

The most outstanding feature of a desmid cell such as *Micrasterias* is its symmetry. There is a difference between bilateral symmetry in relation to a longitudinal axis and bipolar symmetry due to the similarity of both semicells. The latter feature might be regarded as the result of normal cell division and it is easily disturbed by external agents, while bilateral symmetry involves an internal basic feature which is kept intact even if the external shape of the cell should be changed owing to environmental conditions. Species of *Micrasterias*, being unicellular plants with a regular form, seem to be particularly suitable for investigations into the conditions determining structural development. There is the important question as to whether the cytoplasm possesses hereditary properties determining the form independently of the nucleus. In the present work some findings are dealt with which seem to have a bearing upon the nucleo-cytoplasmic relationship.

A Defect Mutation

In cultures of *Micrasterias Thomasiana* there appeared spontaneously nine years ago a defective form which has since then been cultivated as a clone. In fresh cultures the cells are relatively big and exhibit the shape of a half moon, lacking the lateral lobes of one side (plate, fig. 20). The form is

very sensitive to external agents. When the cultures are getting old or conditions have otherwise become unfavourable, the defect mutation clone shows a great diversity of forms, some of which do not differ much from normal cells, while others might resemble, in extreme cases, small moths with wings diverging at different levels. The defective cells exhibit a marked tendency to double side lobes, especially the upper ones (plate, fig. 1—4). In subsequent generations some of the cells might reassume the normal form which will then be maintained constantly in their offspring, the great majority of cells giving rise continuously to defective daughter-cells. Thus a permanent alteration of the form has taken place, and we may use the term defect mutation irrespective of its causal explanation. Consequently its return to the normal form is called remutation.

Under favourable conditions the defective cells often measure about $240\ \mu$ in length and $125\ \mu$ in width, the maximum dimensions of the normal form being about 240 and $220\ \mu$ respectively (cp. Waris 1950). Under unfavourable conditions the size may be more or less diminished. In the chromosome number no difference could be established between the defective and normal form. For the observation of nuclear behaviour the defect mutation is useful because it often shows the nucleo-cytoplasmic surface clearly, while in normal cells this is concealed on both sides by the chloroplast.

It is very probable that the defect mutation is due to a permanent alteration in a cytoplasmic framework serving as a basis for the bilateral symmetry which is not wholly controlled by the nucleus. Thus a cytoplasmic mutation is involved. Such a form does not seem to have been observed earlier (cp. Lefevre 1939).

Denucleated Cells

For many questions concerning the functions of nucleus and cytoplasm, experiments with cells deprived of a nucleus are indispensable. Their importance was already emphasized by Klebs (1887) who made the first detailed study on such plant cells. The classical works by Gerassimoff (1891, 1893, 1897, 1900) and van Wisselingh (1909) contain many admirable observations and are still worthy of attention. The results of older investigations until the year 1936 have been summarized by Küster (1936), who also contributed to the question with personal observations. Most of the studies so far made refer to multicellular plants except those by Hämmerling (1934 a—d, 1943) and Beth (1943) with the uninucleate species of *Acetabulariaceae*, which in regard to their high organization more resemble multinucleate plants. Typical unicellular green plants were first denucleated by Kallio (1949) when he was producing polyploid forms of *Micrasterias*.





Fig. 1—4. *The defect mutation of Micrasterias Thomasiana var. notata*. About 140—130 \times . Centrifuged from 13 to 20 minutes after nucleolar mobilization. Fig. 1 1h 21m, fig. 2 1h 59m, fig. 3 4h 1m, fig. 4 19h 17m from the end of centrifuging. In fig. 3 and 4 a the doubling of the upper side lobes of the new semicells visible (in two planes).

Fig. 5—9. *M. Thomasiana var.*, normal strain. Cell centrifuged 10—20 minutes from nucleolar mobilization. About 130 \times . Fig. 5 1h 6m, fig. 6 1h 35m, fig. 7 2h 51m, fig. 8 3h 41m, fig. 9 7h 46m from the end of centrifuging. Fig. 9 a the binucleate, 9 b the denucleated daughter-cell.

Fig. 10—15. *The defect mutation of M. Thomasiana var.* About 130 \times . Centrifuged at metaphase 19 minutes at 2000 r.p.m. The septum developed after centrifuging has caused a constriction of the chloroplast of which a part is transmitted from one to the other daughter-cell. Fig. 10 39m, fig. 11 1h 32m, fig. 12 2h 14m, fig. 13 4h 10m, fig. 14 8h 12m, fig. 15 22h 40m after centrifuging. In fig. 14 the binucleate daughter-cell full-grown, its new semicell with a new-formed double upper side lobe (close to the denucleated daughter-cell). The new semicell of the denucleated cell only with one polar lobe (short) and both the lateral lobes of one side. In fig. 15 the denucleated daughter-cell alone. The new semicell greatly enlarged, its cytoplasm foamily vacuolate except for a dense part in the middle.

Fig. 16. *M. rotata* at full metaphase (living). About 170 \times .

Fig. 17—19. *M. rotata* centrifuged at metaphase. About 136 \times . Fig. 17 1h, fig. 18 2h, fig. 19 7 $\frac{1}{2}$ h after centrifuging.

Fig. 20. A typical defect mutation cell of *M. Thomasiana var.* About 170 \times .

Enucleate cells or parts of protoplasts have been stated to arise under the following conditions:

a) Spontaneously in thread-like Conjugatae (Gerassimoff 1891, Küster 1936).

b) Experimentally:

1. By expressing protoplasm from cells: Schmitz with *Valonia* (1879, according to Klebs 1887); Palla (1890) with pollen tubes; Acqua (1891, 1910) with pollen tubes; Townsend (1897) with protonema, prothallium, hairs of higher plants.
2. By low temperature: Gerassimoff (1893, 1900) with *Spirogyra*, *Zygnema*, and *Sirogonium*; Kallio (1949) with *Micrasterias*.
3. By high temperature: Kallio (1949) with *Micrasterias*.
4. By plasmolysis: Klebs (1887) with *Zygnema*, *Spirogyra*, *Oedogonium*, and *Funaria*; Palla (1890, 1906) with leaf cells, root hairs, *Oedogonium*, *Marchantia* rhizoids; Pfeiffer (1930) with leaf cells of *Helodea densa*.
5. By narcotic substances: Gerassimoff (1897) with *Spirogyra*; Wada (1940) with hair cells of *Tradescantia*.
6. By centrifuging: van Wisselingh (1909) and Hoffmann (1927) with *Spirogyra*; Malkovský (1923) with *Basidiobolus ranarum*; Küster (1936) with *Mougeotia*; Kallio (1949) with *Micrasterias*.
7. By cutting large cells into pieces; Townsend (1897) with pollen tubes; Hämmerling (1934 a—d, 1943) and Beth (1943) with *Acetabulariaceae*.

In the present investigation a great number — by now over a thousand — cells of *Micrasterias Thomasiana* and *M. rotata* have been denucleated by centrifuging. Whereas at the beginning of the experiments by Kallio and the author this only succeeded occasionally, with the new centrifuge tubes (cp. Waris 1950) and more experience it has later been successful in most cases.

The most favourable instant for centrifuging is about 6—10 minutes after nucleolar mobilization (cp. Waris 1950, p. 4), *i.e.* during metaphase, and a suitable speed is 1500—2500 r.p.m. with a radius of 12.5 cm. The centrifuging is to be continued until the chloroplast of one semicell forms a protuberance of about equal length and width into the isthmus, which is to be checked at intervals of some minutes. Usually it will take 5 to 10 minutes. The longitudinal axis of the cell must lie as close as possible to the centrifugal radius. After anaphase has begun the protoplasm is strongly stiffened and the nuclear spindle is very difficult to displace by centrifuging.

Species of *Micrasterias* are particularly suitable for denucleating by centrifuging owing to their median constriction (cp. Kallio 1949). If the centrifuging has been successful, the protuberance of the chloroplast will be

drawn back and a septum is formed in the isthmus before the spindle has resumed its original position. This results in the formation of two daughter-cells, one of which receives both the daughter-nuclei while the other is left without. It may even happen that the protuberance of the chloroplast is constricted by the new septum and a part of the chloroplast is thus transmitted from one to the other daughter-cell. Both daughter-cells form new semicells, but while those of the binucleate daughter-cells become about normal, in the denucleated ones their shape is much more simple but characteristic of the species in question (plate, fig. 5—9, 17—19). In *M. Thomasiana* it is usually five-lobed but often only three-lobed corresponding to the five or three main lobes respectively, while in *M. rotata* never more than three lobes are formed. If we follow the development in nucleate and denucleated daughter-cells, we see that in the case of three lobes these correspond to one polar lobe and two undivided side lobes, while in the case of five lobes the side lobes are divided into upper and lower ones. Thus the latter have a common basic structure and we can regard the three or five main lobes as homologous parts in the different species.

When cells belonging to the defect mutation are denucleated, the new semicells become unilaterally lobed too (plate, fig. 10—15). Often only the polar lobe and one side lobe will be formed. Sometimes the polar lobe will not bulge out, the new semicell then assuming the form of a bag protruding sideways.

The centrifuging may sometimes result in a disturbance of the symmetry and in this case both the daughter-cells develop abnormally irrespective of whether they have got one nucleus, two nuclei, or none. Such cells are usually not capable of reproduction and die sooner or later.

Discussion

We have seen that in the denucleated cells of *Micrasterias* the new semicells become either bilaterally or unilaterally lobed corresponding to the symmetry relations of the old semicells. These findings must in any case be regarded as full evidence for the conclusion that the symmetry relations of the new semicell — whether bilateral or unilateral — are predetermined at that moment when the dividing cell has formed the septum. Taking into consideration that the septum might even cross the protuberance of the chloroplast caused by centrifuging, the possibility is excluded that the predetermination of symmetry is due to nuclear action, because that part of the cytoplasm which surrounds the nucleus is displaced together with the nucleus (plate, fig. 10—12). We have also seen that the symmetry might

be disturbed despite the fact that the nucleus is present. Thus we must conclude that there is in the cytoplasm of *Micrasterias* and probably even in other desmids a basic framework responsible for the bilateral symmetry and not directly controlled by the nucleus. The cytoplasmic framework of the old semicell seems to differentiate the outer layer of the new semicell at an early stage, as the latter develops in the normal way even if at the stage of a rounded bulge it should become filled with cell sap, the cytoplasm forming only a thin layer adhering to the membrane. This thin cytoplasmic layer which is first differentiated may be identical with the cortex in the sense outlined by Monné (1948). An organized cytoplasmic framework seems very possible if we imagine the living protoplasm in general as consisting of a molecular network (cp. Frey-Wyssling 1948). In *Micrasterias* it seems to presuppose a system of cytoplasmic fibrils arranged symmetrically with a longitudinal axis corresponding to the polar lobes and lateral axes corresponding to the side lobes. This view can, in fact, be supported by some direct observations, for in certain cases when the cell has died while the new semicell has still the form of a rounded bulge, this might show a striation of the cytoplasm radiating from the isthmus towards the membrane, an observation that would be expected if the assumption of a cytoplasmic framework is correct. The three or five main lobes appearing in the new semicells during their development could be thought of as corresponding to relatively important cytoplasmic axes antecedent to the differentiation of form, and even this assumption is not without some evidence in the microscopical observations.

The bilateral symmetry of the basic framework of the cytoplasm is of such fundamental significance that important elements of one side when lost, as in the defect mutation, cannot easily be replaced by elements of the other side nor formed anew by nuclear action. The remutation shows, however, that recovery sometimes occurs, and usually in such a way that the defective cells when dividing immediately form bilateral semicells. There even appear in the defect mutation clone of *M. Thomasiana* forms in which the lateral lobes of one side are only partially lacking. Such variability is understandable if cytoplasmic alterations have occurred.

The above view of the physical architecture of the cytoplasm in desmids may be compatible with the conception of the plasmon by F. von Wettstein (1937) but not with that of plasmagenes in the sense of particles like those of nuclear genes exhibiting their action by chemical influence. It might be questioned whether the form-determining action of the cytoplasm in desmids as exhibited by the defect mutation of *M. Thomasiana* is to be regarded as a case of »Dauermodifikation» (cp. Jollos 1921). The defect mutation is not caused by a treatment, the effect of which should be gradually weakened after the return to normal conditions as seems to be

presupposed for »Dauermodifikation». Supposing that the changed conditions in old cultures where the defect mutation clone has originated may be regarded as »treatment», a regular change of the nutrient solution will not, however, favour the remutation, on the contrary under such good conditions the defect mutation clone shows its most typical cell form. The conception of »Dauermodifikation» seems to imply a substance which can be thought of as becoming diluted during the transmission to subsequent generations. In the alteration of the physical architecture of *Micrasterias* there is nothing that could be diluted.

According to F. von Wettstein (1937) a distinction is to be made between the cytoplasm and the plasmon and the term dauermodification should be referred to the cytoplasm, not to the plasmon, which is placed on a level with the genome. The plasmon is thought of as an internal basis for the genome action resulting in the formation of prerequisites for further genome action. In this sense the supposed cytoplasmic framework in desmids seems to be consistent with the conception of the plasmon, but the distinction between plasmon and cytoplasm seems in the case of desmids to be unreasonable. On the contrary, the cytoplasmic framework must be considered as an integral part of the cytoplasm itself. The form of the cell may be more or less differentiated by the action of the nucleus and dependent on the influence of external agents, but the basis of the bilateral symmetry is always present independently of nuclear action.

Cytoplasmic inheritance, questioned earlier (*e.g.* East 1934), has been accepted as a fact by several authors (*e.g.* Harder 1927, Sirks 1938, Darlington and Mather 1949, Sonneborn 1950, Schultz 1950). On the basis of the available literature it seems that inheritance of a cytoplasmic architecture in plants such as is exhibited by *Micrasterias* has not so far been taken into consideration. Maybe the inheritance of the difference between dextral and sinistral snails assigned by Conklin (1903, according to Sirks 1938) to the structure of the undivided egg-cell is to be regarded as a similar example. Essential for the hypothesis of cytoplasmic inheritance in desmids is the interpretation that the defect mutation is due to a permanent alteration of the cytoplasmic structure. It would be interesting to know whether or not the defective character is preserved during sexual reproduction but unfortunately it has not been possible to induce the cultivated species to reproduce in this way. Presumably it is not preserved. Nevertheless it is not decisive for the validity of the hypothesis. A definitive answer is to be expected from future investigations.

In this connection it is worth noting a striking similarity between the symmetry relations of the defect mutation of *Micrasterias* and species of *Closterium*, the main characteristic in both being a unilateral development

in width and a curvature of the median axis. One would be inclined to believe that the *Closterium* species could have originated from bilaterally symmetrical ancestors through cytoplasmic mutation. Such an assumption would imply an inheritance of this cytoplasmic mutation even during sexual reproduction for which there is so far no proof, and, moreover, remutations would be almost excluded. Thus the question remains unanswered.

The view of desmids as a group of organisms in which the nucleus is not the sole form-determining factor is at variance with the results obtained by Hämmerling with the *Acetabulariaceae* in which the determination of form is only ascribed to substances produced by the nucleus. The desmids seem to represent organisms in which the complete symmetry with regard both to a longitudinal and a transverse axis has been an obstacle to the evolution of more highly differentiated forms. Possibly the extremely small total mass of the chromosomes amounting in *Micrasterias* at metaphase to not even so much as a hundredth of the spindle or nucleus, might in some respect be regarded as a sign of a relatively restricted significance of nuclear functions, in the same way as for instance the giant chromosomes of the salivary glands of dipterans correspond to increased nuclear activities.

Summary

A defective form of *Micrasterias Thomasiana* var. *notata* lacking the lateral lobes of one side has spontaneously appeared in culture about nine years ago and has since then been cultivated as a clone. It is interpreted as due to a cytoplasmic mutation. Certain cells of the offspring might remutate to the normal form.

Cells of *Micrasterias* denucleated by centrifuging are capable of developing new semicells, the form of which is very simple but characteristic of the species in question. In denucleated cells from the normal strains of *M. Thomasiana* the new semicells become symmetrically five- or three-lobed, in *M. rotata* never more than three-lobed. In denucleated cells from the defect mutation clone of *M. Thomasiana* the new semicells become unilaterally lobed corresponding to the symmetry relations of the old semicells.

Centrifuging might sometimes result in disturbing the symmetry in the new semicells of both daughter-cells irrespective of whether they have received one nucleus, two nuclei, or none.

All observations are consistent with the view that there is in the cytoplasm of *Micrasterias* and other desmids a basic framework which, independently of the nucleus, determines the bilateral symmetry of the new semicells developing after cell division, the degree of differentiation, however, being

dependent on nuclear action. Thus a type of cytoplasmic inheritance residing in the cytoplasmic architecture is suggested.

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References

- Acqua, Camillo: Contribuzione alla conoscenza della cellula vegetale. — *Malpighia* 5: 3. 1891.
 — Sulla formazione della parete e sull' accrescimento in masse di plasma prive di nucleo. — *Annali di botanica* 8: 43. 1910.
- Beth, Kurt: Ein- und zweikernige Transplantate verschiedener Acetabulariaceen. — *Die Naturwissenschaften* 31: 206. 1943.
- Darlington, C. D. and Mather, K.: *The Elements of Genetics*. — London 1949.
- Frey-Wyssling, A.: *Submicroscopic Morphology of Protoplasm and its Derivatives*. — New York, Amsterdam, London, Brussels, 1948.
- Gerassimoff, J.: Einige Bemerkungen über die Function des Zellkerns. — *Bull. Soc. Imp. Nat. Moscou* 4: 548. 1891.
 — Ueber die kernlosen Zellen bei einigen Conjugaten. — *Ibidem*, 6: 111. 1893.
 — Ueber ein Verfahren kernlose Zellen zu erhalten. — *Ibidem*, 3: 477. 1897.
 — Ueber die Lage und die Function des Zellkerns. — *Ibidem*, 2 & 3: 220. 1900.
- Hämmerling, Joachim: Über formbildende Substanzen bei *Acetabularia mediterranea*, ihre räumliche und zeitliche Verteilung und ihre Herkunft. — *Arch. für Entwicklungsmechanik der Organismen* 131: 1. 1934 a.
 — Über Genomwirkungen und Formbildungsfähigkeit bei *Acetabularia*. — *Ibidem*, 132: 424. 1934 b.
 — Regenerationsversuche an kernhaltigen und kernlosen Zellteilen von *Acetabularia Wettsteinii*. — *Biol. Zentralblatt* 54: 650. 1934 c.
 — Entwicklungsphysiologische und genetische Grundlagen der Formbildung bei der Schirmalge *Acetabularia*. — *Die Naturwissenschaften* 22: 829. 1934 d.
 — Entwicklung und Regeneration von *Acetabularia crenulata*. — *Z. für ind. Abst. u. Vererbungslehre* 81: 84. 1943.
- Harder, Richard: Zur Frage der Rolle von Kern und Protoplasma im Zellgeschehen und bei der Übertragung von Eigenschaften. — *Z. für Botanik* 19: 337. 1927.
- Hoffman, Curt: Über die Durchlässigkeit kernloser Zellen. — *Planta* 4: 584. 1927.
- Jollos, Victor: Experimentelle Protistenstudien. I. Untersuchungen über Variabilität und Vererbung bei Infusorien. — *Arch. für Protistenkunde* 43: 1. 1921.
- Kallio, Paavo: Artificially produced binuclear, diploid and anuclear *Desmids*. — *Arch. Soc. Zool. Bot. Fenn. »Vanamo»* 2: 42. 1949.
- Klebs, Georg: Ueber den Einfluss des Kernes in der Zelle. — *Biol. Centralblatt* 7: 161. 1887.
- Küster, E.: Über kernlose Zellen. — *Cytologia* 7: 264. 1936.
- Lefevre, M.: *Recherches expérimentales sur le polymorphisme et la tératologie des Desmidiées*. — *Encyclopédie biologique* XIX. Paris 1939.
- Malkovský, C. M.: The Effect of Centrifugal Force upon the *Basidiobolus ranarum* Eid. — *Věstník 1. Sjezdu československých botaniků v Praze*, p. 99. Praha 1923.
- Monné, Ludwik: Functioning of the Cytoplasm. — *Advances in Enzymology* 8: 1. 1948.
- Palla, Ed.: Beobachtungen an des Zellkernes beraubten Protoplasten. — *Flora N. R.* 48: 314. 1890.
 — Über Zellhautbildung kernloser Plasmateile. — *Ber. deutsch. bot. Ges.* 24: 408. 1906.

- Pfeiffer, Hans: Experimentelle und theoretische Untersuchungen über die Entdifferenzierung und Teilung pflanzlicher Dauerzellen. II. Verschieden gerichtete Permeabilitätsveränderungen während der Abgliederung kernloser Zellen aus Dauerelementen des Blattes von *Helodea densa* Casp. — *Protoplasma* 10: 253. 1930.
- Schultz, Jack: The Question of Plasmagenesis. — *Science* 3: 403. 1950.
- Sirks, M. J.: Plasmatic inheritance. — *The Botanical Review* 4: 113. 1938.
- Sonneborn, T. M.: The dependence of the physiological action of a gene on a primer and the relation of primer to gene. — *The American Naturalist* 79: 318. 1944.
- Townsend, Ch. O.: Der Einfluss des Zellkerns auf die Bildung der Zellhaut. — *Jahrb. wiss. Bot.* 30: 484. 1897.
- Wada, Bungo: Experimentelle Untersuchungen lebender Zellen in der Teilung. IV. Die Einwirkung des Dampfgemisches von Ammonia und Chloroform auf die Mitose bei den *Tradescantia*-Haarzellen. — *Cytologia* 10: 158. 1940.
- Waris, Harry: Cytophysiological studies on *Micrasterias* I. Nuclear and Cell Division. — *Physiologia Plantarum* 3: 1. 1950.
- Wettstein, F. von: Die genetische und entwicklungsphysiologische Bedeutung des Cytoplasmas. — *Z. ind. Abst. u. Vererbungslehre* 73: 349. 1937.

Induction Phenomena in Photosynthesis III

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Introduction

In the literature on photosynthesis there exists already for many years a disagreement whether an outburst of CO_2 in the first moments of illumination really occurs. Emerson and Lewis (1) discovered this »gush» in 1941; Emerson and Nishimura (2) confirmed it again in 1949 after its existence had been ignored by Warburg (3). If there is really such a phenomenon as a CO_2 gush, then the criticism of several authors on the quantum-yield determinations of Warburg, must be considered of great importance.

In our experiments with higher plants (4, 5) we never found the slightest indication of such a CO_2 gush; we found on the contrary a rather strong initial uptake of CO_2 after the beginning of an illumination.

Warburg and also Emerson c.s. worked with the alga *Chlorella pyrenoidosa*, so it remained possible that this alga behaved in a different way than higher plants. We hardly expected it to be different, however, because Aufdemgarten (6, 7) examined the two algae *Stichococcus bacillaris* and *Hormidium flaccidum* and found for both the same behaviour as was recorded for higher plants.

These two algae belong to the Ulotrichales while *Chlorella* is a representative of the Chlorococcales. Therefore we wanted to investigate *Chlorella* especially but we also used *Chlorococcus olivaceus* (belonging to the same group as *Chlorella*) and *Ulothrix* sp. (belonging to the same group as *Hormidium* and *Stichococcus*).

Method

For the apparatus used we refer to our previous papers (4, 5); the change in CO_2 content of the gas passing the assimilation chamber was determined

by recording changes in the heat conductivity of the gas. In air, N_2 and O_2 only CO_2 changes were recorded; in hydrogen also changes in O_2 could easily be determined.

At first we tried to have the gas bubbling through a dense suspension of the algae; this technique, however, was not altogether satisfactory. The water of the suspension acted too much as a buffer, so that quick differences in gas exchange were so much flattened out, that we could not get a clear picture of the induction line of photosynthesis in these algae.

Much better results were obtained when a very thick suspension of centrifuged algae was spread over a wet piece of the filterpaper of which three cm^2 were put into the assimilation chamber and treated like leaf laminae. In this way we got recordings which gave a satisfactory induction curve.

The *Chlorella*'s were grown in Warburg's inorganic medium. The culture was illuminated by fluorescent light of $\pm 40,000$ ergs/ cm^2 /sec and grown in flasks of the model described by Benson c.s. (8) for continuous cultivation of *Chlorella*. Every three days a sample of the algae was centrifuged, spread over filterpaper and put into the assimilation chamber for examination. This chamber consisted of a flattened tube of approximately $50 \times 20 \times 3$ mm. Through this chamber the gas passed at a speed of ± 2 l/h. Usually the gas contained $\pm 3\%$ CO_2 .

Experimental part

The adaptation curve of *Chlorella* showed indeed a course that was strongly different from the adaptation curve in higher plants. The alga reacts to a sudden illumination with a release of a gas, heavier than N_2 (fig. 1). That this gas is CO_2 was made probable by the fact that the initial gush dis-

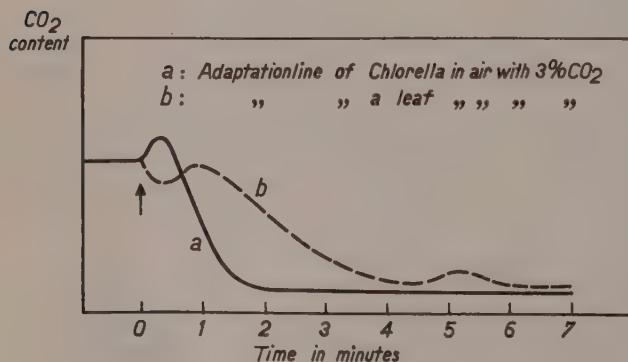


Fig. 1. (a) Adaptationline for *Chlorella* showing an initial outburst of CO_2 .

(b) Adaptationline of a leaf of *Dahlia* showing an initial CO_2 uptake, which is normal in leaves of higher plants.

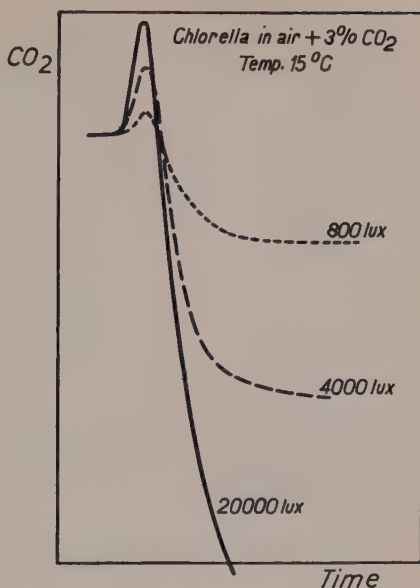


Fig. 2. The CO₂-»gush» of Chlorella at different light-intensities.

appeared completely when the air stream, after passing the assimilation chamber but before entering the measuring bloc, was led over Ba(OH)₂.

As far as we could investigate Chlorella always reacted to an illumination with this »gush». Only after a few moments this gush is followed by a CO₂ consumption, which is normal in photosynthesis.

Another difference between Chlorella-adaptation and adaptation of higher plants, is that Chlorella during long periods of darkness is only slightly deadapted, while higher plants after a long dark period always need a long time before they are adapted to the illumination given.

So it seems that photosynthesis in Chlorella proceeds along a slightly different path than in other plants. When *Protococcus olivaceus* was examined, we found exactly the same adaptation curve as in Chlorella. On the other hand *Ulothrix* behaved like higher plants and like *Hormidium* and *Stichococcus*.

These differences show that probably the pattern of the photosynthesis in the Chlorococcales is rather different from that in Ultrichales and in higher plants.

In our previous studies (4, 5) we analysed the effect of different environmental influences on the adaptation line of higher plants. In this paper the effect of these influences on the adaptationline of Chlorella is studied.

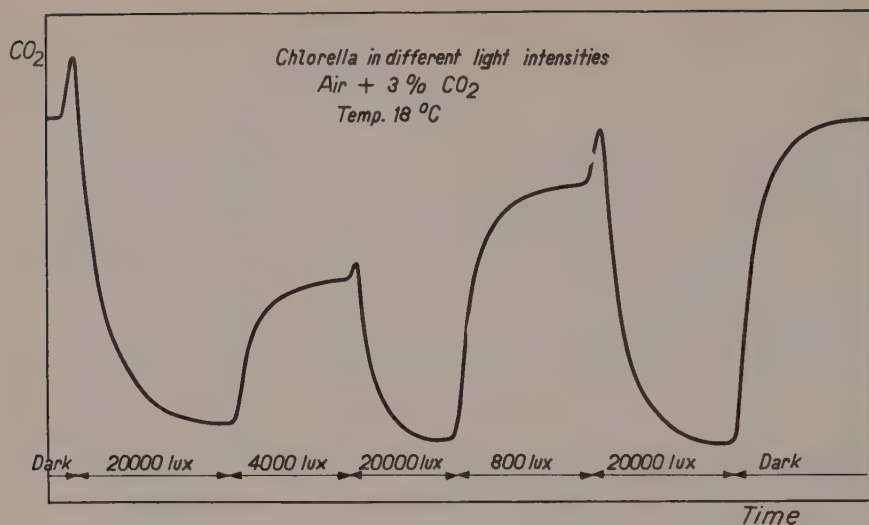


Fig. 3. The CO₂-line of *Chlorella* during changes from low to high light-intensities.

Light intensity

As might be expected the gush increased with increasing light intensity. In strong light (20,000 lux from a high pressure mercury lamp, type HO 2000) the gush was much stronger than at 4,000 lux; at 800 lux the gush was still smaller (fig. 2). A gush appears when changing from low to high light intensity (fig. 3).

Temperature

With increasing temperature the gush diminishes to disappear almost completely when the temperature reaches more than 27° C (fig. 4). Of course it is quite possible that the gush fundamentally still exists at higher temperature, but then it is completely masked by the immediate start of a strong photosynthesis so that all CO₂ that is released, is assimilated at the same moment, so that it can not be shown in the surrounding gas.

At 20° C the gush always appears clearly at the beginning of an illumination; it becomes gradually stronger when the temperature is lowered down to 10° C. At still lower temperature the gush remains constant.

Preceding dark period

The duration of the preceding dark period has a great influence. After longer dark periods the gush will be larger. A maximum is reached only

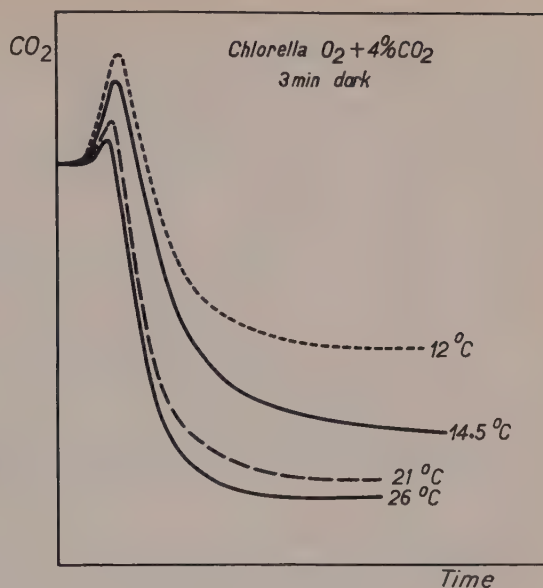


Fig. 4. The influence of different temperatures on the CO₂-gush.

after several hours. It was not well possible to determine the influence of the dark period exactly because the intensity and the duration of the illumination before the dark period too was important. Long periods of strong illumination before a dark period of 4 minutes resulted in a smaller gush than shorter illumination periods did (fig. 5).

Duration of the gush

To determine the length of time necessary for the gush to complete itself a series of experiments were done with illuminations of a few seconds only. At a temperature of 17° C the gush was less than maximal when the illuminations lasted less than 8 seconds. The dark period before the illumination was 4 minutes in every case. So we must conclude that under these circumstances the gush is complete in 8 seconds.

Is the gush really a photochemical effect?

When a piece of wet filterpaper is put in the assimilation chamber the CO₂ dissolved in the water of this filterpaper will soon be in equilibrium with the CO₂ in the passing air stream. If the temperature is suddenly raised

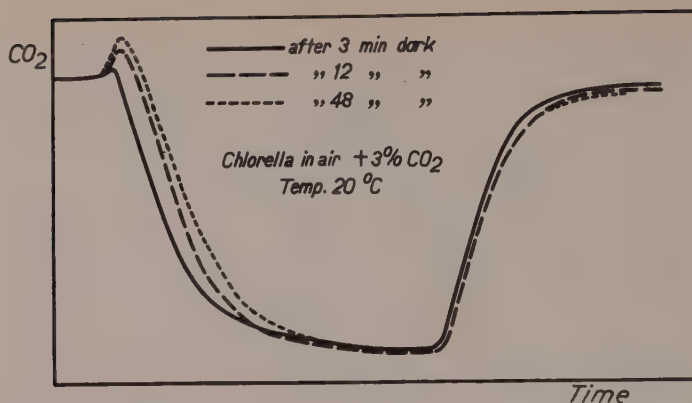


Fig. 5. The influence of the duration of the preceding dark-period on the light-adaptation-line of *Chlorella*.

by a few degrees CO_2 will be quickly released until a new equilibrium is reached at this higher temperature. If the illumination caused an increase in temperature in the filterpaper with *Chlorella*s on it, an effect would be found very similar to this »gush». That the illumination would cause such a difference in temperature was, however, highly improbable because the mercury lamp was burning under water and the assimilation chamber was kept in a thermostate under water too. We could easily show that the gush was a real photoeffect by exposing a filterpaper with *Chlorella*, which had given a very clear gush at 17°C to 55°C for a few minutes. After this treatment they did not show any »gush» when examined again at 17°C .

Experiment in O_2 , N_2 and H_2 atmosphere

In oxygen + 3 % CO_2 the algae behaved in exactly the same way as in air. The gush was as clearly visible, decreased with increasing temperature, increased with longer dark periods and increased with higher light intensity.

In pure oxygen without CO_2 no gush could be found. In darkness there remains a small CO_2 production which disappears when the algae are illuminated.

In hydrogen + 3 % CO_2 a slightly different induction line was produced. The gush was lower and was quickly followed by a second top, after which normal adaptation took place (fig. 6). As was already mentioned in a medium of hydrogen changes of O_2 and of CO_2 both will be registered by the apparatus, so the line shown in fig. 7 a might be partly derived from

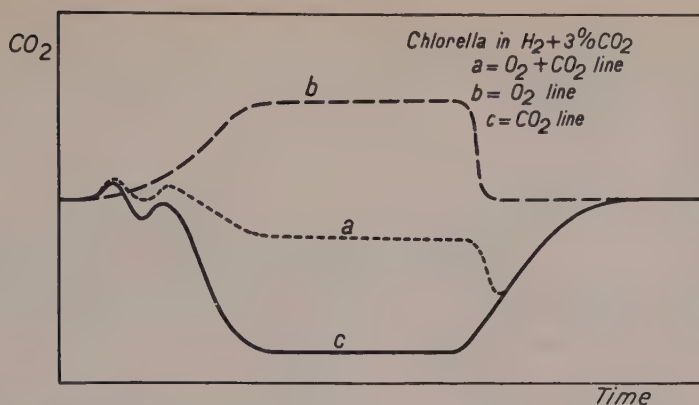


Fig. 6. *Light-adaptation-line of Chlorella in hydrogen with 3% CO₂. a: line indicating the heat conductivity changes of the gas due to combined variations in CO₂ and O₂ during the induction period. b: line indicating changes in O₂-content. c: line indicating changes in CO₂-content.*

changes in O₂ production. By letting the gas pass through Ba(OH)₂ the CO₂ was taken away and only changes in O₂ remain. From the O₂ line (fig. 7 b) which shows no special peaks or other irregularities, it is obvious that the two peaks in line *a* are caused by changes in CO₂ content.

Examining the first of these two peaks we found that it is obviously the same thing as the «gush» in air+3 % CO₂. It is higher at higher light intensities, it is lower at higher temperatures, it is higher after longer dark periods.

The second peak, however, is different. We did not find anything like it with Chlorella in other gases. There is a possibility that it is caused by the same factor as the initial CO₂ uptake of higher plants (1) because after longer dark periods this peak appears later.

At the end of an illumination the O₂+CO₂ line in H₂+CO₂ often steeply goes down before coming up to the zero-line. This is caused by the fact that almost immediately after darkness no O₂ is produced anymore, while CO₂ is still assimilated until an equilibrium is reached with the CO₂ content of the gas. Fig. 6 shows the course of the O₂ line, the CO₂ line and the combined line, which last one is measured when no absorbing apparatus is used between the assimilation chamber and the measuring bloc.

The combined line (fig. 6 a) shows a delayed uptake of CO₂ after the end of the illumination which is of course the same effect as has already been noted by McAlister (9) and which he called the «pick up».

In pure H₂ without any CO₂ no «gush» exists. During illumination a small amount of O₂ is released; in darkness a little bit of CO₂ is constantly produced.

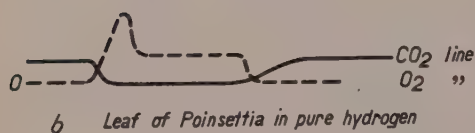
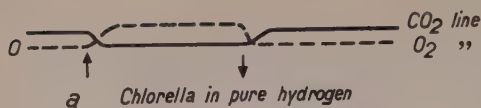


Fig. 7. *Chlorella* (a) and *Poinsettia* leaf (b) in hydrogen without CO_2 show differences in O_2 release when exposed to light.

It is rather remarkable that in light the oxygen is released and not used up for respiration. It seems that not only inside the chloroplasts but also inside the whole cell the oxygen which originates from photosynthesis, exists only as some organic peroxide, but not as dissolved free oxygen. Probably only at the cell surface the oxygen is released as O_2 and then the greater part of it, will be taken away by the passing stream of gas.

The adaptation line of higher plants in pure H_2 or N_2

When leaves of higher plants are examined in pure hydrogen without CO_2 they respond to illumination in a rather different way from that of *Chlorella* (fig. 7 b). Their first response is a »gush» of gas but in this case not an outburst of CO_2 but of O_2 .

With the light intensity used (20,000 lux from a high pressure mercury lamp type HO 2000) temperature had no influence on this O_2 gush as long as it remained below 25°C . At higher temperatures the O_2 gush decreased slowly but gradually. At 38°C it amounted to less than half of the value which it had below 25°C . This decrease of the O_2 gush at higher temperature was totally reversible; when returning to 20°C after an examination at 31°C , the O_2 gush was exactly the same as before the examination at 31°C .

The preceding dark period had also some influence on the O_2 gush, but only when at 18°C it lasted shorter than 2 minutes. After a dark period of 2 minutes or more the O_2 gush is maximal.

The light intensity correlated strongly with the O_2 gush. At 20,000 lux the gush was very clear while at 800 lux hardly any gush could be measured.

The continuous O_2 release in light of 20,000, 4,000, and 800 lux were the same. An interesting fact, however, could be noted when changing the light intensity from 20,000 to 4,000 or to 800 lux. In those circumstances a decrease

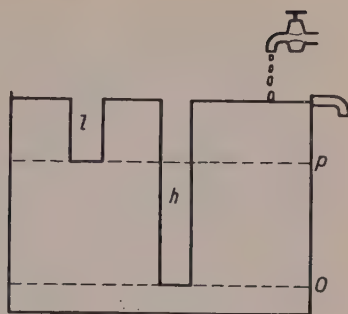


Fig. 8. Diagram showing the constant production of CO_2 in the cell and the reduction of this CO_2 by different intensities of light (see text).

in O_2 release occurred during a short interval; after a few minutes, however, the old level was reached again.

From these experiments one gets the impression that in darkness some reducible substance accumulates up to a certain level, which at room temperature is reached in about 2 minutes. This most probably is CO_2 . If then the illumination begins this amount of CO_2 is quickly reduced, a process which causes the O_2 gush. Afterwards only the gradually produced CO_2 can be used for reduction; this corresponds to the continuous appearance of O_2 during illumination.

At high light intensities the CO_2 produced will be immediately reduced; therefore the CO_2 level in the cell will be very low. At lower intensities the reducing power will not be so great. Then the CO_2 level can rise a little higher. Only after a minute or more this new level will be reached and only then all the CO_2 produced will be used for photosynthesis. This must be the reason why, when lowering the light intensity in the first few moments no O_2 will be produced and only later on the O_2 release will be equal to that under high light intensity.

In a diagram it can be shown as in fig. 8. A continual filling of the basin A through the tap shows the continual production of CO_2 in the cell. If a gap (h) is opened in the wall of the basin through which the water can flow out, the water level in the basin will go down to level o . This shows the reducing power of the chloroplasts at high light intensity which keeps the CO_2 down to a low level. The gap l stands for the much less reducing power at low light intensity. If gap h is closed and l opened the water will first have to rise to level p before it flows out of the gap. In darkness both gaps are closed and the water will flow out at the rim after it has risen to this level, which means that the CO_2 will be released only when this level is reached. For this same reason after the end of an illumination no CO_2 will appear in the passing air stream during the first few moments.

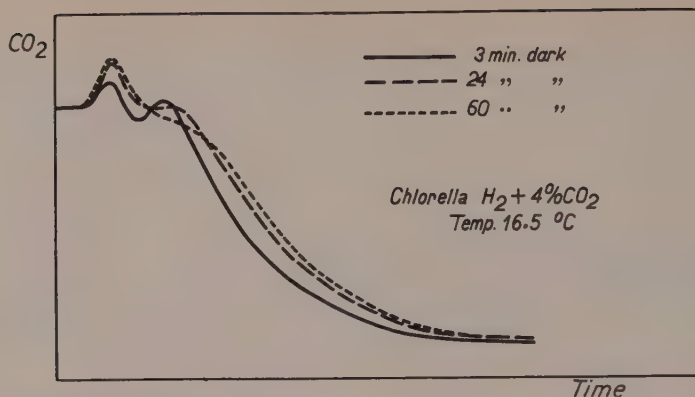


Fig. 9. Adaptation-line of *Chlorella* in hydrogen + 4 % CO_2 after different dark-periods (CO_2 -line).

After two minutes of darkness, however, in our experiments the CO_2 release will be normal; most probably that will be the reason why only after two minutes of darkness the O_2 gush will be maximal. In our diagram in fig. 8 this means that the O_2 gush corresponds to the release of water through gap *h* when this gap is suddenly opened, and the water level in the basin comes down to level *o*.

Adaptation time in *Chlorella*

In higher plants we noted that the duration of the preceding dark period had a very strong effect on the adaptation time to a strong illumination. With *Chlorella* this was much less so. A dark period of 3 minutes gave a line that was only a very little bit steeper than a dark period of one hour (fig. 9). Even 16 hours of darkness resulted only a small increase on the amount of time necessary for complete adaptation.

Summary

It was shown that the reaction of *Chlorella* to sudden illumination is much different from that of higher plants.

While higher plants always begin with an initial uptake of CO_2 , *Chlorella* does exactly the opposite and begins with a release of CO_2 before it starts photosynthesizing.

This gush of CO_2 is positively correlated with light intensity, negatively with temperature and positively with the duration of the preceding dark period.

Another difference between higher plants and *Chlorella* is the deadaptation in darkness. In *Chlorella* there is not much difference in adaptation time after short and long dark periods. In higher plants, however, after long periods of darkness adaptation takes much longer than after short periods of darkness.

A third difference is the behaviour in pure hydrogen without any CO_2 . In this environment higher plants react to illumination with a »gush» of oxygen, which we could not find when examining *Chlorella*.

So *Chlorella* has a rather different pattern of photosynthesis than higher plants. *Protococcus olivaceus* Rabenh., which like *Chlorella* belongs to the Protococcales, behaves in the same way as *Chlorella*. *Ulothrix sp.*, like *Horridium flaccidum* and *Stichococcus bacillaris*, all three belonging to the Ulotrichales, have induction lines like higher plants.

References

1. Emerson, R. and Lewis, C. M.: Carbon dioxide exchange and the measurement of the quantum yield of photosynthesis. — Am. J. Bot. 26: 808. 1939.
2. Emerson, R. and Nishimura, M. S.: Photosynthesis in plants. — Monograph of the Am. Soc. of Plant Physiologists ed. by J. Franck and W. E. Lewis, p. 219. 1949.
3. Warburg, O.: Assimilatory quotient and photochemical yield. — Am. J. Bot. 35: 194. 1948.
4. van der Veen, R.: Induction phenomena in photosynthesis I. — Physiologia Plantarum 2: 217. 1949.
5. — Induction phenomena in photosynthesis II. — Physiologia Plantarum 2: 287. 1949.
6. Aufdemgarten, H.: Zur Kenntnis der sogenannten Induktionsvorgänge bei der Kohlensäureassimilation. — Planta 29: 643. 1939.
7. — Weitere Untersuchungen mit dem Gaswechselschreiber über die Kohlensäureassimilation. — Planta 30: 343. 1939.
8. Benson, A. A., Calvin, M., Haas, V. A., Aronoff, S., Hall, A. G., Bassham, J. A., and Weigl, J. W.: Photosynthesis in Plants. — Monograph of the Am. Soc. of Plant Physiologists ed. by J. Franck and W. E. Loomis, p. 381. 1949.
9. McAlister, E. D.: The chlorophyll-carbondioxide ratio during photosynthesis. — J. Gen. Physiol. 22: 613. 1939.

Growth Studies in Woody Species I. Photoperiodism in First-Year Seedlings of *Pinus silvestris*

By

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The existence of a capacity for photoperiodic response in woody species was first shown by Garner and Allard (1923). Since then a number of further instances of photoperiodism in woody species has been reported, the subject having recently been reviewed by the writer (Wareing, 1949 a). In the vast majority of woody species so far investigated it is found that short days (of the order of 10 hours' duration) reduce the period of extension-growth and hasten the onset of dormancy, as indicated by the formation of resting-buds and the onset of leaf-fall (in deciduous species). With long photoperiods the period of extension growth is increased, and in some species e.g. *Liriodendron tulipifera* (Garner and Allard, 1923) and *Robinia pseudacacia* (Kramer, 1936) there appears to be a certain 'critical' photoperiod (Wareing, 1949 a) above which growth may be maintained for at least 18 months. In other species, however, e.g. *Fraxinus americana*, *Quercus borealis* (Kramer, 1936), *Acer pseudoplatanus* (author, unpublished) there appears to be no critical photoperiod above which growth may be maintained indefinitely, and in such species dormancy is not appreciably delayed, even under continuous light, as compared with normal 'long days' (although short days bring hastened dormancy). In several instances (e.g. Kramer, 1937) these photoperiodic effects have been evoked by quite low light-intensities, just as is the case with the flowering response in herbaceous species (Withrow and Benedict, 1936). This fact suggests that the mechanism of photoperiodism may be the same in both woody and herbaceous species, although the nature of the response is different. Nevertheless, intensive studies on the mechanism of photoperiodism in woody species have not pre-

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viously been carried out, although information on this subject would be of considerable interest in supplementing the detailed studies carried out with herbaceous plants.

The work to be described here forms part of a detailed investigation of the phenomena associated with photoperiodism in a single woody species, *Pinus silvestris*. The occurrence of photoperiodic phenomena has been reported for a number of species of *Pinus* (Kramer, 1936; Jester and Kramer, 1939), and it has been shown that 'short days' hasten and 'long days' delay the onset of dormancy in seedlings, but detailed studies are not reported. The occurrence of photoperiodic effects in *P. silvestris* has been reported by the writer (Wareing, 1949 b) in a preliminary note, and has been confirmed for first-year seedlings by Karschon (1949), in respect of hypocotyl length and weight of leaves.

The present paper deals with photoperiodism in first-year seedlings only, and comprises the first part of a thesis accepted for the degree of Ph. D. in the University of London.

First-year seedlings of *P. silvestris* differ in two important respects from older plants of this species, viz. (1) The green leaves are 'juvenile' leaves borne on the main stem and are 'determinate' in growth, as contrasted with the paired 'needles' of indeterminate growth and borne on 'dwarf-shoots', of older plants. (2) The duration of growth of the shoot in first year seedlings is not predetermined by the number of initials already laid down in a resting bud, as it is in the older stages (Wight, 1933). In the development of a seedling, emergence is followed by a period of leaf-formation not accompanied at first by any extension of the internodes, so that a 'rosette' of leaves is formed. This phase occupies 6—8 weeks, after which extension of the internodes commences between the leaves at the base of the rosette. From then onwards there is a continuous extension of successive internodes between the basal leaves of the rosette and this process keeps pace with the continued production of new leaves at the apex, so that a rosette of leaves continues to exist in the apical region throughout growth. This phase of growth ultimately ceases with the formation of a terminal resting bud at the centre of the apical rosette of leaves, which remains present throughout the period of dormancy.

Experimental

Experiment 1. Effect of long and short photoperiods

Methods. The experimental plants were grown in the open in pots (diameter 9 cms.) containing soil obtained from Wareham Forest, Dorset, to which had been added 25 % by volume of a suitable compost, each pot containing 5—6 seedlings. On 15th June 50 pots of seedlings were placed in each of two light-proof boxes, constructed of a wooden framework and covered with roofing felt. Each box was provided with a detachable lid which was removed for a specified period each day. One series (hereinafter referred to as 'S.D.') was exposed in this way to a 10-hour

Table 1. *Effect of short and long photoperiods on growth of first-year seedlings.*

Series	Mean duration of growth (days)	Mean number of leaves	Mean length of stem (cms)	Mean length of first 25 internodes (mm)	Leaf length (cms)
S.D.	84	56.2 ± 2.6	1.41 ± 0.08	0.32 ± 0.02	2.4 ± 0.04
L.D.	112	86.4 ± 2.5	4.20 ± 0.20	0.60 ± 0.02	3.0 ± 0.04

(In this and all later tables the Mean \pm Standard Error is quoted.)

photoperiod (from 6 a.m. to 4 p.m.) and the other ('L.D.') to a 15-hour photoperiod (from 5 a.m. to 8 p.m.). From 8th August it was necessary to provide supplementary illumination for the 'long day' plants from a 100-watt lamp giving a light-intensity at the soil level of approximately 20 foot-candles. Samples consisting of the plants from 5 pots were taken from each series for dry-weight determination at intervals of 2 weeks throughout the growing period. Observations were also made on the mean stem length and number of leaves for each plant of these samples throughout the growing period.

Results. The normal pattern of growth described above was followed by both 'S.D.' and 'L.D.' seedlings. In the 'S.D.' series, however, dormancy

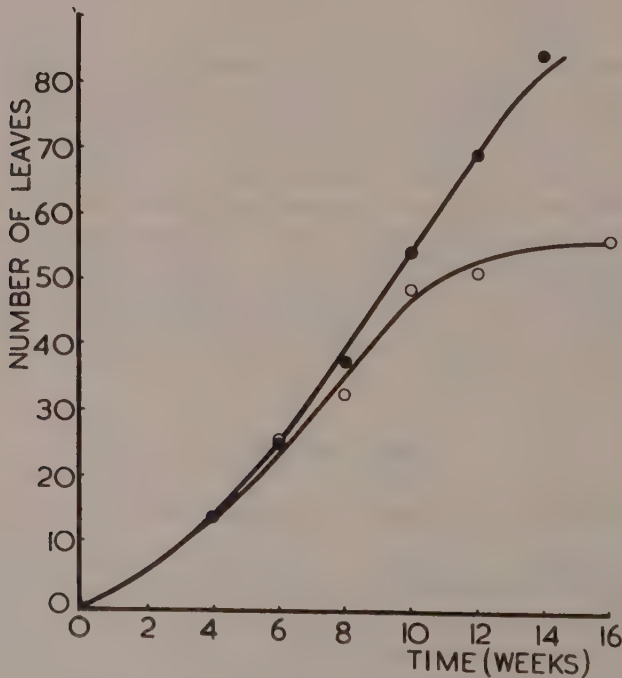


Fig. 1. *Leaf-development in seedlings grown under 10-hour (—○—) and 15-hour (—●—) photoperiods respectively, during the course of Experiment 1.*



Fig. 2. First-year seedlings of *Pinus silvestris* grown under 10-hour (left) and 15-hour (right) photoperiods respectively.

(as indicated by the formation of terminal resting-buds) commenced 4 weeks earlier than in the 'L.D.' series. The data for the mature seedlings are given in Table 1. It is seen that there is a significant difference in total leaf-number between the two series, but the observations on the progressive increase in leaf-number between the two series indicated that this difference in final leaf-number was due mainly to the difference in *duration* of growth, there being little difference between the rates of leaf-production in the two series (Fig. 1). The difference in mean length of stem, however, was partly due to the difference in the number of internodes and partly to the fact that the internodes were much shorter in the S.D. plants. This difference is clearly seen in Figure 2.

The mean length of the leaves was also significantly less in the S.D. plants. The question as to whether these are true photoperiodic effects, as opposed to effects arising from differences in assimilation, is considered below.

From the samples taken at 2-weekly intervals the increases in dry-weight of root and shoot throughout the period of growth were determined. The relative growth of shoot and root is frequently measured by the so-called »shoot/root ratio«, but a more satisfactory criterion is the 'differential growth coefficient' (Huxley, 1932). Pearsall (1927) has shown that for many plants there is a linear relation between the logarithms of the shoot- and root-weight viz.

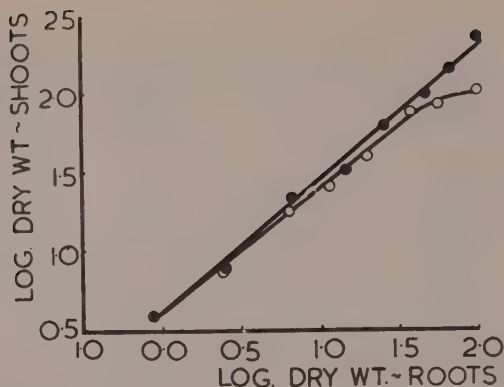


Fig. 3. Relative growth of shoot and root in seedlings grown under 10 hour (—○—) and 15 hour (—●—) photoperiods respectively over the period of Experiment 1. (Calculated regression coefficients for log. shoot weight on log. root weight are 0.819 and 0.749 for the L.D. and S.D. series respectively).

$$S = cR^k \quad (1)$$

or $\log S = k \log R + c$
 where S = shoot weight
 R = root weight

and k , c are constants. The value of the 'differential growth coefficient' is then given by k .

Figure 3 shows the results of plotting log shoot-weight against log root-weight for the two series of plants of the present experiment. It is seen that the rule holds closely for both L.D. and S.D. plants during the period of active growth of the shoot, but there is deviation from the linear relation in the later stages of the S.D. series due, of course, to the cessation of growth of the shoot, while root-growth continued. The straight portions of both L.D. and S.D. curves are practically parallel (the difference between the regression coefficients for log shoot-weight on log root-weight for the two series is not significant); that is to say, the value of k , (given by the slope of the curve) is little affected by the length of the photoperiod. Thus under the conditions of this experiment, the relative growth of shoot and root was little affected by the length of the photoperiod.

Experiment 2. Effect of continuous illumination

The following experiment was carried out in order to determine whether or not there is a certain 'critical' length of photoperiod, above which growth may be maintained indefinitely, in the case of seedlings of *P. silvestris*.

Table 2. *Effect of continuous illumination as compared with natural photoperiods (May—September).*

Treatment	No. of plants	Mean length of stem (cm)	Length of internode (mm)	Mean number of leaves	Mean duration of growth (days)
Natural photoperiods	47	3.47 ± 0.13	0.86	64.7 ± 2.0	116
Continuous illumination	49	5.90 ± 0.23	1.37	53.4 ± 1.6	103

Methods. Two series of plants, grown in the open in pots as in the previous experiment, were used. One series was exposed to natural day-length conditions during the period of growth (early May to mid-September) and the other was exposed to continuous illumination by supplementing the natural photoperiod by artificial illumination at approximately 25 foot-candles from sunset to sunrise.

Results. A greater rate of extension-growth soon became apparent in the plants exposed to continuous illumination, but instead of dormancy being delayed as compared with that in plants grown under natural photoperiods, it was actually hastened, the mean duration of growth being 13 days shorter under continuous illumination. Although the total number of leaves was less under continuous illumination (Table 2), the length of the stem was greater, due to the very much longer internodes than under natural photoperiods. Other differences shown by the plants under continuous illumination were: (1) There was no terminal 'rosette' of leaves, nearly all the internodes being fully extended. (2) The lateral 'dwarf shoots' grew out in the axils of the juvenile leaves in the terminal region. (3) The terminal buds were very much smaller in size than those of the 'natural day length' series. Since these results were produced by illumination at only 25 foot-candles, they must be regarded as true photoperiodic effects.

Experiment 3. Interruption of the Light- and Dark-Periods

One of the characteristic features of photoperiodism in herbaceous species is that although the interruption of the photoperiod by a period of darkness usually has no effect, interruption of the dark period by a short 'light-break' may completely nullify the effect of the dark period. As these effects do not appear to have been reported previously for woody species, the following experiment was carried out.

Methods. The culture methods were the same as in the preceding experiments. The plants were divided into four series and exposed to day-length conditions as follows:

Series A. Exposed to a 15 hour photoperiod, (from 6 a.m. to 9 p.m.), using

Table 3. *Effect on growth of interrupting (a) the photoperiod, (b) the dark period (see text for details).*

Series	No. of plants	Mean length of stem (cm)	Length of internode (mm)	Mean number of leaves	Mean duration of growth (days)
A	52	4.62 ± 0.21	1.07	66.1 ± 2.1	119
B	47	1.51 ± 0.08	0.62	38.2 ± 0.9	86
C	54	5.69 ± 0.20	1.00	79.1 ± 1.8	135
D	50	3.54 ± 0.11	0.99	49.3 ± 1.7	95

natural daylight, supplemented as necessary by artificial illumination at 20 foot-candles.

Series B. Exposed to an 11-hour photoperiod (6 a.m. to 5 p.m.) using only natural daylight.

Series C. Exposed to two periods of light of 7 and 4 hours' duration respectively, (a) from 6 a.m. to 1 p.m.; (b) from 5 p.m. to 9 p.m. Artificial illumination at 20 foot-candles was used to supplement the second period of illumination as necessary, as for series A. The conditions were thus identical with those for series A, except that the photoperiod was interrupted by 4 hours' dark from 1—5 p.m.

Series D. Exposed to 10 1/2 hour photoperiod from 6 a.m. to 4.30 p.m. and to 30 minutes artificial illumination at 20 foot-candles from 8.30—9 p.m. The conditions were thus the same as for series C, except that the dark period was interrupted by a light-break after 4 hours' darkness.

Results. The data for the mature plants are summarised in Table 3. The plants of series A and B showed the 'long day' and 'short day' types of response observed in the two previous experiments. The plants of series C also showed the 'long day' type of response, and indeed the duration of growth, leaf-number and height at maturity were greater than with series A. Thus interruption of the light period by a 4-hour dark period did not interfere with the typical 'long day' response, but indeed resulted in enhanced long-day characteristics. This latter result was somewhat unexpected and was the subject of further investigation which is described below.

The plants of series D, which received a light break of only 30 minutes' duration at 20 foot-candles, showed a significantly greater total leaf-number and internode length than the 'short day' plants of series B, but did not attain the leaf-number of the plants of series A. Thus interruption of the dark period by a 'light break' significantly affected the response and tended to reduce the short-day effects resulting from a long (unbroken) dark period, just as in herbaceous species.

Experiment 4. Optimum Photoperiod for Extension Growth

Among the results of the two preceding experiments were the observations that (1) a greater leaf-number is attained under natural 'long-day' con-

Table 4. *Effect of different photoperiods on growth of seedlings.*
(Data for 30 plants under each photoperiod.)

Photoperiod	10 ¹	12	14	16	18	20	22	24
Total stem length (cms)	{ 0.76 ± 0.05	1.06 ± 0.06	1.50 ± 0.09	1.78 ± 0.10	4.10 ± 0.20	6.32 ± 0.23	3.91 ± 0.20	3.68 ± 0.17
Total no. of leaves	{ 36.7 ± 1.53	44.9 ± 1.95	53.2 ± 2.3	61.6 ± 1.3	71.9 ± 2.2	74.7 ± 2.7	51.4 ± 2.1	50.6 ± 1.6
Leaves in terminal rosette...	—	—	29.2	28.8	19.5	7.7	11.5	11.6
No. of extended internodes	—	—	24.0	32.8	52.4	67.0	39.9	39.0
Internode length (mm)...	—	—	0.466	0.413	0.716	0.906	0.935	0.889
Mean duration of growth (days)	90	87	94	102	129	151	96	87

¹ Data for 10-hour day plants of Experiment 6 described below.

ditions than under continuous illumination; (2) seedlings which received a photoperiod interrupted by 4 hours of darkness in addition to a 9-hour dark period each day attained a greater leaf-number than plants which received only one period of darkness of 9 hours' duration per day. These two observations suggested that a high leaf-number is favoured by a short period of darkness, and that if two such dark periods are given per 24-hour cycle, then their effects may be cumulative. In order to test this hypothesis the following two further experiments were carried out, the first of which consisted of growing seedlings under a range of photoperiods from 12 hours to continuous illumination, in order to determine under what duration of dark-period the maximum leaf-number is attained.

Methods. The plants were grown under the same conditions as previously. On emergence on 12th June they were divided into 7 different series each containing approximately 50 seedlings in 10 pots, and were exposed to photoperiods of 12, 14, 16, 18, 20, 22 and 24 hours, respectively. In the case of the 12, 14 and 16 hours photoperiods the full photoperiod consisted of natural day-light (except for a short period of additional illumination at the end of the growing period in the case of the 16-hour series), while the plants of the remaining series received the full natural photoperiod, supplemented by artificial illumination at 20 foot-candles to give the required photoperiods. A number of plants under the 18 and 20-hour photoperiods had still not completed their growth by October, and these plants were therefore then transferred to a greenhouse maintained at 15° C, until all growth had been completed at the end of November.

Results. The data for the mature plants are summarised in Table 4 and Figure 4. (These data include also a series of plants grown under a 10-hour

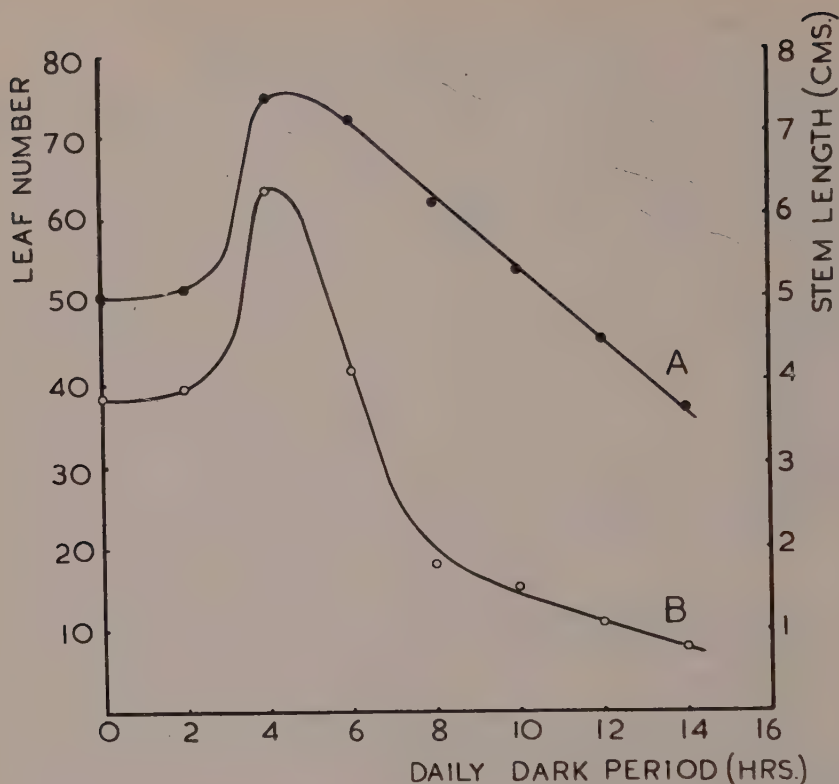


Fig. 4. Variation in leaf-number (A.) and stem-length (B) with length of dark-period.

photoperiod, forming part of Experiment 6 described below, which ran concurrently with the present experiment.)

In Figure 4 the mean leaf-numbers are plotted against the length of the daily dark-period. It is seen that the maximum leaf-number is attained with a daily dark period of 4 hours (20-hour photoperiod), and that with only 2 hours' dark or with continuous illumination the final leaf-number falls off sharply. With dark periods of duration greater than 4 hours, the leaf-number falls linearly with increasing length of dark period. The curve for plant-height is much more »peaky» than that for leaf-number, since total height is a function of the two variables, node (or leaf)-number and inter-node-length. The greatest internode length was at 2 hours' darkness, with only a slightly diminished value at 4 hours' dark and continuous illumination (Table 4).

One of the most notable features of these results is the striking difference between the plants under 22-hour and 20-hour photoperiods respectively



Fig. 5. *Mature seedlings grown under various photoperiods as indicated (Experiment 4).*

(see Fig. 5). The former resembled very closely the plants grown under continuous illumination, which showed the features already noted for the corresponding treatment in Experiment 2, viz. relatively short growing period, reduced leaf-number, high internode length, small terminal buds and premature growth of 'dwarf-shoots' in the axils of the juvenile leaves. The 20-hour day plants, however, showed a very long growing period, markedly higher leaf-number, large terminal buds and very little tendency for the growth of dwarf shoots. At the time the experiment was terminated, a few plants under continuous illumination showed premature breaking of the terminal resting buds and a fresh period of shoot elongation.

A further point of interest is that the number of unextended internodes forming the terminal 'rosette' of leaves was a minimum in the 20-hour day plants, and was greatest with the shorter photoperiods. These differences do not become apparent until the end of the growing period, for all plants, irrespective of the day-length conditions, show a well-marked rosette during the active stages of growth; but whereas under short day conditions leaf-formation and internode extension appear to cease at approximately the same time, under a 20-hour photoperiod the upper internodes continue extending even after the presence of a terminal bud has become apparent and leaf-formation has obviously ceased. It thus appears that for any given daily photoperiod a certain mean number of internodes can undergo extension, and this number does not necessarily coincide with the total number of leaves formed, so that if there are any additional leaves present above the highest extensible internode, these must remain in the form of a 'rosette'. *Thus the apical meristem and the region of internode extension appear to show distinct and independent responses to the length of the photoperiod.*

Table 5. *Effect of 4-hour dark periods on growth of seedlings.*

Series	No. of plants	Mean length of stem (cm)	Mean internode length (mm)	Mean number of leaves	Mean duration of growth (days)
A	30	10.12 ± 0.42	1.125	98.7 ± 4.4	138
B	30	6.32 ± 0.23	0.906	74.7 ± 2.7	151

Experiment 5. Effect of two daily short dark periods

The following experiment was carried out using a further series of plants from the same batch of seedlings as those used in Experiment 4. This experiment was designed to test the second part of the hypothesis put forward above, viz. that a short dark period promotes a high leaf-number, and that if two or more such periods are given per 24-hour cycle, then their effects are cumulative.

Methods. The present experiment ran concurrently with Experiment 4, and (apart from the day-length treatment) the plants were grown under conditions identical with those of the previous experiment. The plants (designated »Series A») were exposed to two daily 8-hour photoperiods. (1) from 3 a.m. to 11 a.m. (2) from 3 p.m. to 11 p.m. These light periods were separated by dark periods, each of 4 hours' duration. Supplementary artificial illumination at 20 foot-candles was given from 3 a.m. to sunrise and from sunset to 11 p.m. The growth of these plants was compared with that of the 20-hour day plants of Experiment 4 (referred to as »Series B»), which were grown alongside the plants of series A. These plants of series B were exposed to a light period from 3 a.m. to 11 p.m., the source of artificial illumination being common to both series A and B. Thus the light conditions were identical for both series, except that the plants of series A received an additional dark period from 11 a.m. to 3 p.m. In October a few plants of series A were still growing and they were transferred to the greenhouse at the same time as the plants of series B (see above).

Results. Growth in the plants of series A proceeded vigorously throughout the period of the experiment, and the plants soon became noticeably taller than those of series B. The data for the mature plants are summarised in Table 5, and corresponding data for the plants of series B are repeated from Table 4 for comparison.

It is seen that although the mean duration of growth was 13 days shorter for the plants of series A, these attained a greater total leaf-number and showed a greater internode length than those of series B. The difference in total height between the two series is clearly indicated in Figure 6.

A point of considerable interest is that a single alternation of 20 hours' light and 4 hours' dark results in an increase in total leaf-number of 24.1 as compared with exposure to continuous illumination (Table 4), and when two cycles of 8 hours' light and 4 hours' dark are introduced into the 24-



Fig. 6. Seedlings grown under (B) one 4-hour dark period, and (A) two 4-hour periods, per 24-hour cycle.

hour cycle there is a further increase in leaf-number by 24.0 (Table 5). The remarkably close agreement between these two figures strongly supports the hypothesis that an increased leaf-number results from a short dark period and that when two such periods are introduced, the effects are cumulative. Since the duration of growth of series A was actually less than that of series B, it is clear that the rates of leaf-production and stem elongation were greatly accelerated by the introduction of two dark periods into the 24-hour cycle.

The dry weights of the shoots and roots were determined for both series when all shoot-growth had ceased. The data (Table 6) show that the mean dry weight of the shoots of series A was greater than that of series B, but the difference was not proportional to the difference in leaf-number and stem-length. The mean dry weight of the roots of series A was *less* than that of series B, however, the roots of the former being very poorly developed. Evidently the additional short dark period stimulated growth of the shoot at the expense of that of roots. The total dry weight for the plants of series A was less than that of series B, a fact which must probably be ascribed to reduced assimilation under the conditions for series A.

Table 6. Dry weight data (Experiment 5).

Series	Mean dry weights			S/R ratio
	Shoot (gms)	Root (gms)	Total plants (gms)	
A	0.217 ± 0.010	0.0517 ± 0.0028	0.269 ± 0.013	4.2
B	0.193 ± 0.010	0.126 ± 0.010	0.319 ± 0.018	1.5

The plants of series A also differed from the majority of plants of series B in that they showed poor development of the terminal bud, accompanied by premature growth of 'dwarf-shoots' in the axils of the leaves of the terminal region.

Experiment 6. Photoperiodic After-Effect

Moshkov (1932) and Scepotjev (1948) have reported the occurrence of 'photoperiodic after-effects' in various woody species. In order to ascertain whether similar effects occur in seedlings of *P. sylvestris* and to obtain more information on this phenomenon, the following experiment was carried out.

Methods. Two series of seedlings, comprising 17 and 18 pots respectively, were used. On emergence of the seedlings, the first series was subjected to a 10-hour day, and the second to a 15-hour day. After 15 days 9 pots were transferred from 15-hour to 10-hour photoperiods. After a further 15 days these latter plants were transferred back to 15-hour days, and at the same time 8 pots which had until then remained continuously in short-days, were transferred to long days. The day-length conditions were then maintained unchanged until all plants had become dormant, when the mean heights and leaf-number of all plants were determined.

There were thus four series of plants, exposed to day-length conditions as follows:

Series A. 10-hour photoperiod throughout.

Series B. 15-hour photoperiod throughout.

Series C. 15-hour photoperiods throughout, except for 15 short photoperiods from 12th to 27th June.

Series D. Exposed to 10-hour photoperiods for the first 30 days from emergence, and then transferred to 15-hour photoperiods.

Results. The data for the four series of plants are summarised in Table 7. Both series C and D, which received periods of exposure to short days, formed fewer leaves than did those which received a 15-hour photoperiod throughout, the differences being highly significant. On the other hand both series C and D formed a significantly *greater* number of leaves than did the plants of series A, which received a 10-hour photoperiod throughout. Now the plants of series D received the same treatment as those of series A for the first 30 days, and hence the difference in leaf-number between these two series must have arisen *after* the date of transfer from short to long days, indicating that a further period of leaf formation must have occurred after that date. Nevertheless the plants of series D had fewer leaves than those of series B, so that the effects of the previous short day treatment became apparent even after a further period of leaf formation under long-days. Hence the occurrence of a true photoperiodic after-effect is established for series D, and a similar conclusion is also indicated for series C.

If we compare the number of leaves (1) on the extended portion of the stem, and (2) in the terminal rosette, in the plants of series C and D with

Table 7. *Photoperiodic after-effect* (see text for details).

Series	No. of plants	Length of stem (cms)	Leaf numbers			Mean duration of growth (days)
			Leaves on extended portion of stem	Leaves in terminal rosette	Total leaves	
A	30	$\begin{cases} 0.76 \\ \pm 0.05 \end{cases}$	—	—	$\begin{cases} 36.70 \\ \pm 1.52 \end{cases}$	77
B	50	$\begin{cases} 1.45 \\ \pm 0.06 \end{cases}$	$\begin{cases} 27.8 \\ \pm 0.7 \end{cases}$	$\begin{cases} 28.3 \\ \pm 0.9 \end{cases}$	$\begin{cases} 56.22 \\ \pm 1.36 \end{cases}$	87
C	49	$\begin{cases} 1.47 \\ \pm 0.07 \end{cases}$	$\begin{cases} 26.2 \\ \pm 1.1 \end{cases}$	$\begin{cases} 21.2 \\ \pm 1.1 \end{cases}$	$\begin{cases} 47.45 \\ \pm 1.65 \end{cases}$	77
D	49	$\begin{cases} 1.39 \\ \pm 0.09 \end{cases}$	$\begin{cases} 24.5 \\ \pm 1.1 \end{cases}$	$\begin{cases} 19.4 \\ \pm 1.1 \end{cases}$	$\begin{cases} 43.86 \\ \pm 1.72 \end{cases}$	77

Critical difference in total leaf-number at 5 % probability:

(1) B—C: 2.14

(2) B—D: 2.16

those grown under long days throughout, it is seen that the differences in respect of the former are only slight, and that the differences in total leaf-number are mainly due to differences in the number of leaves in the terminal rosette. Moreover, there are only slight differences with respect to the length of the extended portion of the stem in the three series in question. These observations agree with the suggestion put forward above (Experiment 4) that under a given length of photoperiod the extension of a certain maximum number of internodes is possible, and that this 'internode response' is independent of the total number of leaves formed. Hence differences in total leaf-number are reflected mainly in the number of leaves forming the terminal rosette.

Discussion

It has been shown above that under a 15-hour photoperiod the duration of the growing period is increased, a greater total number of leaves is formed and the internode length is much greater, as compared with corresponding plants grown under a 10-hour photoperiod. As the length of the photoperiod is still further increased, a maximum for duration of growth and total leaf-number is reached with a daily photoperiod of 20-hours, and with photoperiods greater than this the duration of growth and total leaf-number falls sharply. The occurrence of maximum growth at a photoperiod of less than 24 hours has been reported also for *Salix babylonica* and *Pyrus ussuriensis* by Moshkov (1932).

Although the experimental plants were grown in the open and no attempt was made to control temperature conditions or to ensure that the *quantity* of light received was the same in all the plants of any given experiment, the effects observed cannot be due to differences in assimilation, as is shown by the following considerations.

(1) In a simple experiment (not described above) to test the effect of growing seedlings under muslin so that the intensity of natural daylight was reduced to 50 % of normal, the plants differed little from those grown under full daylight and did not show the type of response observed when plants are grown under short days with full light intensity.

(2) The use of low-intensity illumination to prolong the natural photoperiod had a marked effect on the duration of growth and leaf-number of the plants. For example, the differences between plants under 18 and 20-hour photoperiods (Experiment 4) resulted simply from 2-hours' additional illumination at 20 foot-candles received by the latter. On the other hand, despite the differences in quantity of daylight received by the various series of plants of Experiment 4, there is a very linear relation between the total leaf-number and the length of the photoperiod (Figure 4) over a wide range of different day-length conditions.

(3) Interruption of the dark-period by a short light-break at low intensity produced a significant increase in leaf-number and internode-length.

(4) The increased growth observed in Experiments 3 and 5 when an additional short dark-period was introduced into the 24-hour cycle is actually the reverse of what would be expected from reduced assimilation.

It was suggested above that the occurrence of a maximum leaf-number under a 20-hour photoperiod is due to the fact that short dark periods (up to 4 hours' duration) favour a high leaf-number. This is strongly supported by the observation that (1) a single daily alternation of 20 hours' light and 4 hours' dark results in an increase in total leaf-number of 24.1 as compared with continuous illumination, and that (2) when two cycles of 8 hours' light and 4 hours' dark are introduced into the 24-hour cycle, then there is a further increase in leaf-number by 24.0. The close agreement between the increase in total leaf-number in the two cases suggests that it is the dark period which is the determining factor, and that the length of the light period is unimportant. Nevertheless, it is clear that in order to obtain the growth-promoting effect in the first hours of dark it is necessary that the preceding light period shall be of a certain minimum duration and/or intensity. This is shown by the results of Experiment 3, in which both series C and series D received two daily dark periods of 4 and 9 hours respectively, but whereas in the case of series C the 9-hour dark period was preceded by a 4-hour light period (mainly daylight), in the case of series D the corresponding period of illumination was 30 minutes at low-intensity. The plants of series C showed an increased leaf-number as compared with the plants of series B (which received a single daily dark period of 9 hours), whereas the leaf-number of the plants of series D did not attain

that of the plants of series B. Thus, in order to obtain the growth-promoting effect during the first hours of dark, the preceding period of illumination must have been of a certain minimum duration and intensity, and this requirement is satisfied by a few hours of daylight. This conclusion agrees with the observations of Mann (1940) and others that in order to obtain flowering in short-day herbaceous species, the dark period must be preceded by a light period of a certain minimum intensity and duration. Following Hamner (1942) and Gregory (1948) we may postulate some substance »A» which is formed in the light phase. Since the same increase in leaf-number (24) was obtained with a single dark period of 4 hours, preceded by a photoperiod of 20 hours, as with each of two daily 4-hour dark periods preceded by only 8 hours of light, it would appear that the substance A reaches 'saturation' after a few hours of daylight and that once this occurs, it is the length of the dark period which is the determining factor. This conclusion is also supported by the very linear relation between leaf-number and the duration of dark periods greater than 4 hours (Figure 4), this linearity suggesting that the response is determined by the absolute length of the dark period and is not affected by the duration of the light period (over the range covered by the experiment). This agrees with the observation that in short-day herbaceous species the length of the 'critical' dark period is independent of the length of the photoperiod (see Gregory, 1948), but is difficult to reconcile with the observation that the number of flowers formed is affected by the length of the photoperiod (Hamner, 1940).

The question arises as to why short dark periods (up to 4 hours) promote a high leaf-number, whereas longer dark periods result in a reduced leaf-number. Now, there are good reasons for regarding the cessation of growth of the annual shoot in woody species as an inhibition phenomenon. This is seen not only in the phenomena of 'correlative inhibition' but particularly clearly in those species (e.g. *Tilia europaea*, *Robinia pseudacacia*) with a sympodial growth-habit, in which the apical region is actively abscised when a certain stage of development has been attained. Experiments to be described in a later paper of this series also support this view. Thus, the reduced leaf-number observed under long dark periods is probably to be regarded as due to the fact the action of some inhibitor mechanism is favoured under such conditions.

We are therefore led to postulate two antagonistic systems operating within the plant during the dark period viz. (1) a growth-promoting substance 'A' formed during the preceding light phase, and which is active during the first hours of darkness, and (2) a growth-inhibitor system which becomes effective after 4-hours of darkness. The suggestion is put forward that during the first 4 hours of dark there is still a supply of 'A' available as a carry-

over from the light phase, but that after 4 hours this is exhausted or removed from some field of action, thus permitting the inhibitor-mechanism to come into effect. In order to explain the increased leaf-number resulting from a daily 4-hour dark period as compared with continuous illumination it is necessary to postulate that provided there is still a supply of 'A' available, then dark actually favours its action.

It is clear that if an inhibitor-system favoured by dark determines the cessation of growth under dark periods of greater than 4 hours' duration, then some other mechanism must be postulated to account for formation of terminal resting buds with dark periods shorter than 4 hours. Some evidence for this is provided by the formation of abnormally small buds, together with a loss of apical dominance as shown by the premature growth of dwarf-shoots, under continuous illumination.

The suggestion has been put forward by the writer (1949 b) that photo-periodic effects in woody species result from the effect of light upon the production or availability of auxin within the plant. In a later paper evidence will be adduced for the view that the substance A postulated above is auxin or an auxin-precursor, and that the inhibitor-mechanism which is effective under long dark periods may involve an auxin-inhibitor.

Summary

1. Under a 10-hour photoperiod first-year seedlings of *Pinus silvestris* show the following differences as compared with seedlings grown under a 15-hour photoperiod: (a) the duration of growth is reduced; (b) fewer leaves are formed; (c) the internode-length and leaf-length are reduced. No obvious differences with respect to root-development were observable, and the relative development of root and shoot was not affected by the day-length conditions.

2. If seedlings are grown under a range of different photoperiods from 10 hours to continuous illumination, the maximum height and leaf-number is attained under a 20-hour photoperiod, and with photoperiods greater than this there is a sharp fall in the leaf-number. It is shown that this latter effect is probably due to the fact that short dark periods promote a high leaf-number whereas long dark periods result in a reduced leaf-number.

3. When two 4-hour dark periods are introduced into the 24-hour cycle, the increased leaf-numbers produced by each dark period are summated.

4. The effects produced under short-day conditions are considerably reduced when the dark period is interrupted by a short 'light-break'.

5. It is shown that a true 'photoperiodic after-effect' occurs when seedlings are transferred from short-day to long-day conditions.

6. The apical meristem and region of internode extension appear to show distinct and independent reactions to the length of the photoperiod.

7. The interpretation of these results is discussed and the hypothesis is put forward that two mutually antagonistic systems are operative in seedlings of *P. silvestris* during the dark period: (a) a system promoting continued growth of the shoot, which depends upon some substance produced during the preceding light-phase and which is effective during the first hours of darkness; (b) an inhibitor-system which becomes operative after 4 hours of dark and which results in the earlier cessation of growth in proportion to the length of the dark period.

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References

- Garner, W. W. & Allard, H. A.: Further Studies in Photoperiodism, the Response of the Plant to Relative Length of Day. — J. Agr. Res. 23: 871. 1923.
- Gregory, F. G.: The Control of Flowering in Plants. — S.E.B. Symp. 2: 75. 1948.
- Hamner, K. C.: Interrelation of Light and Darkness in Photoperiodic Induction. — Bot. Gaz. 101: 658. 1940.
- Hormones and Photoperiodism. — Cold. Spr. Harb. Symp. Quart. Biol. 10: 49. 1942.
- Huxley, J. S.: Problems of Relative Growth. — London 1932.
- Jester, J. R. & Kramer, P. J.: The Effect of Length of Day on the Height Growth of certain Forest Tree Seedlings. — Journ. Forestry. 37: 796. 1939.
- Karschon, von R.: Untersuchungen über die physiologische Variabilität von Föhrenkeimlingen autochthoner Populationen. — Mitt. der Schweiz. Anstalt f. d. forstliche Versuchswesen, 26: 205. 1949.
- Kramer, P. J.: The Effect of Variation in Length of Day on the Growth and Dormancy of Trees. — Plant. Phys. 11: 122. 1936.
- Photoperiodic Stimulation of Growth by Artificial Light as a cause of Winter Killing. — Plant. Phys. 12: 881. 1937.
- Mann, K. L.: Effect of some environmental factors on floral initiation in *Xanthium*. — Bot. Gaz. 102: 339. 1940.
- Moshkov, B. S.: Photoperiodicity of Trees and its Practical importance. — Bull. App. Bot., Gen. & Pl. Breeding. Ser. A. Soc. Pl. Ind. 2: 108. 1932.
- Pearsall, W. H.: Growth Studies VI. — On the relative sizes of growing plant organs. Ann. Bot. 41: 549. 1927.
- Scepotjev, F. L.: Doklady Akad. Nauk S.S.S.R. 59: 1201. 1948 (Herb. Abst. 490/48).

- Wareing, P. F.: Photoperiodism in Woody Species. — *Forestry*. 22: 211. 1949 a.
— Photoperiodic Control of Leaf Growth and Cambial Activity in *Pinus silvestris*. — *Nature* 163: 770. 1949 b.
- Wight, W.: Radial Growth of the Xylem and Starch Reserves of *Pinus silvestris*. — *New Phyt.* 32: 77. 1933.
- Withrow, R. B. & Benedict, H. M.: Photoperiodic Responses of Certain Greenhouse Annuals as influenced by Intensity and Wavelength of Artificial Light used to Lengthen the Daylight Period. — *Plant. Phys.* 11: 225. 1936.

Studies on Growth and Metabolism of Roots. IV. Positive and Negative Auxin Effects on Cell Elongation

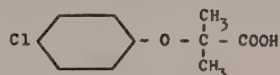
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Introduction

It was shown in a paper by Wilske and Burström (9) that the growth-inhibiting action on roots of phenoxy compounds decreases, if the side chain is blocked off from the ring system by substituting -O- with -SO- or -SO₂-, in accordance with presumptions made by Erdtman and Nilsson (5). Searching for other substances with such blocking bridges in the side chain they have prepared α -(p-chlorophenoxy)-isobutyric acid:



A preparation generously placed at our disposal by Prof. Erdtman has been tested as to its growth effect and influence on the protein metabolism in roots; the results of the former part of the investigation are presented below.

The culture methods have been the same as described in a previous communication (4). The behaviour of the roots was followed microscopically and graphs of the cell elongation together with computations of the rate of cell multiplication within the epidermis were made in the same way as in earlier studies on auxin effects (2, 3).

It should be especially noted that all experiments were carried out under constant conditions, a temperature of 20° C, artificial light, and with flowing nutrient solutions. Thus the concentration of the solutions was as constant as practically possible, which is of importance for the results. A change in pH could not be avoided; the initial value amounted to about 4.5, and the final pH to 5.0—5.8. This rather high

rise partly depended upon vigorous aeration of the culture vessels, which reduced the buffering action through carbon dioxide. Plants were examined at 48 hour intervals, after 2, 4, and 6 days of treatment.

In the text below α -(p-chlorophenoxy)-isobutyric acid is abbreviated P.C.I.B. Comparative experiments were carried out with β -indolylacetic acid, called auxin.

General appearance of the plants

One series of experiments with additions of P.C.I.B in the concentrations 10^{-4} , 10^{-5} , and 10^{-6} mol will be reported in detail as an example. Figure 1 shows the root elongation. In 10^{-4} mol the roots died, in 10^{-5} there was only a slight growth after the second day. In 10^{-6} mol, on the contrary, the root elongation was increased by about 60 per cent during the first two days and somewhat less up to the sixth day. This increase was observed in every instance in the presence of P.C.I.B in concentrations from $3 \cdot 10^{-7}$ to $3 \cdot 10^{-6}$ mol and it is higher and more consistent than any other growth accelerations of roots observed with compounds of the auxin type. It was always highest in the first interval of time and fell gradually, an example of the common phenomenon of adaptation to the growth compounds (4). Sometimes the average elongation of the roots at the start was more than double the normal rate, the figure 60 per cent represents the lowest figure observed.

Figure 2 visualizes the appearance of such root systems; in this instance

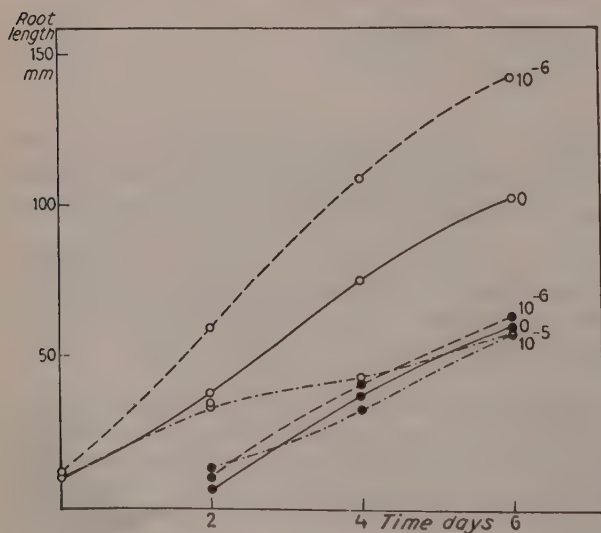


Fig. 1. The influence of P.C.I.B on the root elongation. Concentration in mol. Open circles denote R_{I-III} , filled circles R_{IV-V} . — Figs. 1 and 3—7 illustrate one series of experiments.

not only the three first roots, R_{I-III} , but also the second pair of adventitious roots, R_{IV-V} , is accelerated. This was usually the case, and the conditions shown in figure 1 are exceptional.

Under the microscope these rapidly growing roots appeared entirely normal. Lateral roots were initiated in a regular sequence, but they developed more slowly than in the control plants. The P.C.I.B roots completely lacked root hairs, but this is a usual consequence of an increased cell elongation. In 10^{-5} mol, however, a very peculiar phenomenon appeared. As will be more fully discussed below and is visible from figures 4 and 5 the toxic action depends upon a reduced cell multiplication, but the change in the individual cell length is small and sometimes it increases. Nevertheless, the cells lack root hairs, and, furthermore, the whole roots are twisted so that the epidermal cell rows, instead of running straight longitudinally, form spirals with up to 30° deviation from the axis. Unfortunately it was very difficult to obtain clear pictures of this phenomenon. Moreover, there appear, on close inspection, small

intercellulars between the epidermal cells, a condition encountered previously in connexion with growth disturbances (3).

As to the action on the leaf development the acid is, of course, much less active (figure 3). Any accelerating actions are lacking in the concentrations tested, the difference between 10^{-6} mol and the control is statistically insignificant. Then there is a gradually increasing growth inhibition, much less than with roots, and the leaves were viable even in 10^{-4} mol. In short, the reaction of the shoots presents the picture to be expected as a consequence of the reduced or inhibited root growth in the higher concentrations.



Fig. 2. The shape of roots grown in P.C.I.B solution. To the right control roots, to the left roots from $3 \cdot 10^{-6}$ mol P.C.I.B. Average roots after 48 hours' treatment.

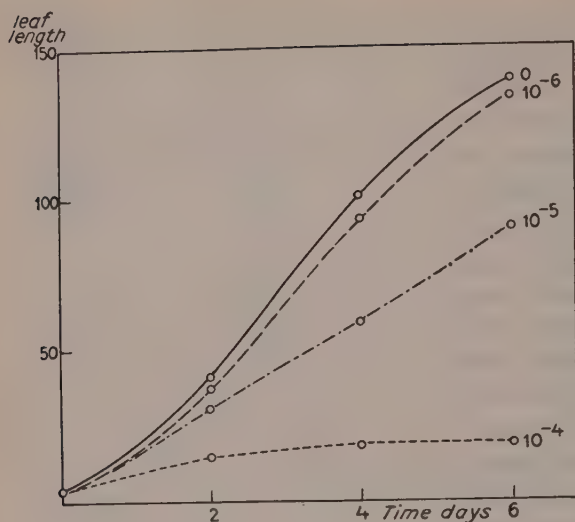


Fig. 3. The influence of P.C.I.B. on the elongation of the first green leaf. — Cf. figs. 1, 4—7.

Analysis of the growth reaction

In the usual way (2, 3, 9) the total elongation of the roots was divided into the multiplication of the cells in the meristem and the growth by cell stretching. The result from the previously quoted experiment is shown in figures 4 and 5. The picture is very clear but some comments are necessary. *The accelerated growth wholly depends upon an increased length of the individual cells whereas the cell number is unaltered.* Even in 10^{-5} mol there is a significant increase in cell length and the retardation of the gross root growth depends upon the reduced cell multiplication. This is apparently a toxic effect apart from the action on cell elongation; it is further a cumulative action as seen from the time course of the curve for 10^{-5} mol in figure 5. As to the cell lengths there is no difference between R_{I-III} and R_{IV-V} as shown in figure 4. This was confirmed throughout the experiments.

Concerning the general course of the growth curves with time a peak was constantly formed between the second and fourth day, just as in earlier series (9). This is apparently due to some shift in the general organization of the plant, the decrease from the fourth to sixth day might, for instance, be caused by the incipient formation of laterals and the increasing development of R_{IV-V} . Nevertheless, due attention has to be paid to this trend in evaluating the effects of P.C.I.B.

It was also observed, that the average cell length is more constant than the number of cells formed. This is illustrated by table 1, showing the figures for the second interval of control roots from six consecutive series

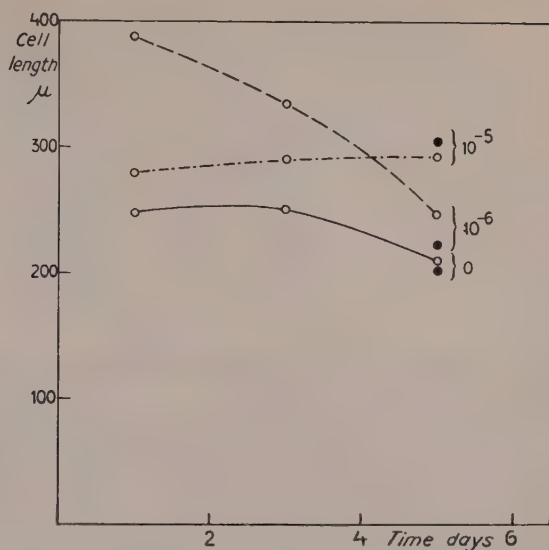


Fig. 4. Lengths of epidermal cells on roots in P.C.I.B solutions. Concentrations of P.C.I.B given in mol. Open circles denote R_{I-III} , filled circles R_{IV-V} . Cf. figs. 1, 3, 5—7.

of experiments, arranged according to increasing root length. It was further found that the treatment with P.C.I.B may decrease or increase the cell number irregularly, with an average not differing from that of the control, whereas the increase in cell length was fairly constant. In four experiments with $3 \cdot 10^{-6}$ mol P.C.I.B the increase in cell length during the first interval amounted to 140, 137, 143, and 170 μ respectively.

This means that it is more correct to express the action on the elongation mechanism simply in terms of the cell length, disregarding accidental

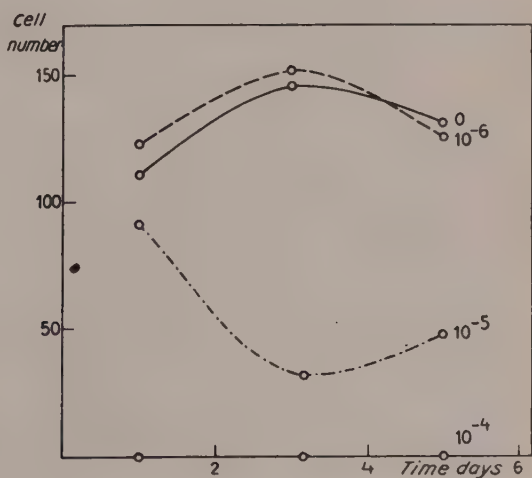


Fig. 5. The number of epidermal cells of roots in P.C.I.B. — Cf. figs. 1, 3, 4, 6—7.

Tab. 1. *The relation between individual cell length and rate of cell multiplication. Figures from control roots in six series of experiments, 2nd interval of time.*

Increase in root length mm	Cell length μ	Cell number
33	267 ± 6	124
36	258 ± 6	140
37	251 ± 6	146
40	268 ± 6	147
44	256 ± 6	173
51	271 ± 6	188

variations in the cell multiplication, provided that this does not differ too much from the control.

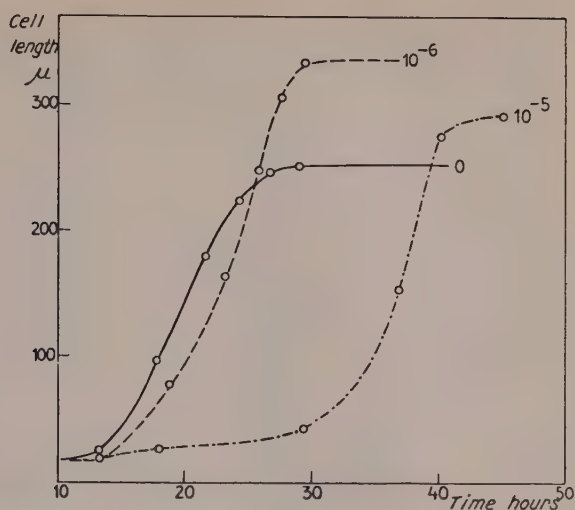
Another point illustrated in figure 4 is that the acceleration through P.C.I.B decreases with the duration of the treatment both absolutely and relatively when compared with the control. On an average the increase in cell length during the second and third 48 hour intervals amounted to 89 and 87 μ respectively in 6 series of experiments against 140—170 μ for the first interval. This is an obvious case of adaptation to the compound. In another instance (4) such an adaptation was shown to depend upon a specific sensitivity of cells in actual progress of stretching at the initial addition of the compound. Such an explanation is inadequate in the present case.

After it was shown that the accelerating effect of P.C.I.B on root growth is due to an action on the cell elongation the growth curves for the cells were constructed in several experiments. They all agreed fairly well in their main features, and as an example the graphs for interval 2 from the experiment recorded in figures 1 and 3 to 5 are presented in figure 6.

These S-shaped curves show that the elongation is delayed slightly in 10^{-6} mol and very much in 10^{-5} when compared with the control. A further analysis is possible by construction of the grand periods of elongation (figure 7) from the values in figure 6.

As regards 10^{-6} mol, which deserves the most interest, the curve follows that for the control plants at the start of the elongation. Then, however, it appears as if the cells, instead of passing the peak of the curve, received a new incitement to elongation, the rate rising to a value of twice the normal one. Owing to the rapid decline of the rate again, the total duration of the elongation remains nearly the same as in the control, or about 19 hours. Although only one series of observations is presented here, this picture can be generalized because the plants reacted remarkably uniformly. It ought to be stressed that an increase in root length of not more than 50 per cent, as in this case, reveals an actual increase in the maximal rate of elongation of the individual cell of about 100 per cent.

Fig. 6. Graphs showing the elongation of epidermal cells of roots in P.C.I.B. — Concentrations in mol. Cf. figs. 1, 3—5, 7.



This doubling of the rate of elongation occurred remarkably constantly in 10^{-6} or $3 \cdot 10^{-6}$ mol P.C.I.B., no consistent order of activity being established for these two concentrations. Figure 8 shows another set of graphs, which have been arranged so that the start phases coincide. The acceleration again appears as an addition to the second half of the grand period, and the time of elongation still attains a value between 18 and 20 hours.

It is striking that P.C.I.B. in two respects has the opposite effect on the cell elongation as that of auxin (2). Auxin (β -indolylacetic acid) was shown to accelerate the first phase of the cell elongation and shorten the second

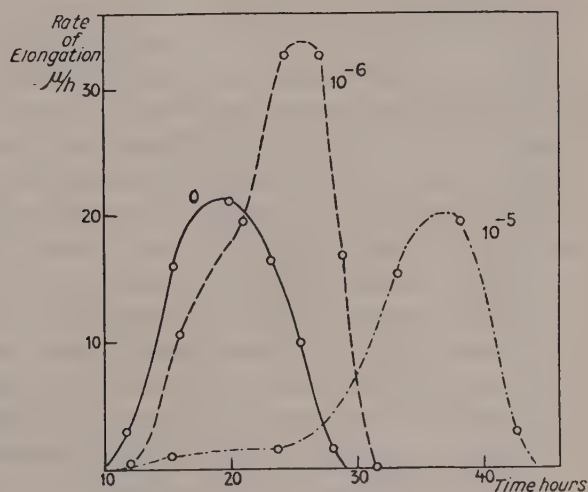


Fig. 7. The grand periods of elongation of epidermal cells of roots in P.C.I.B. — Computed from fig. 6.

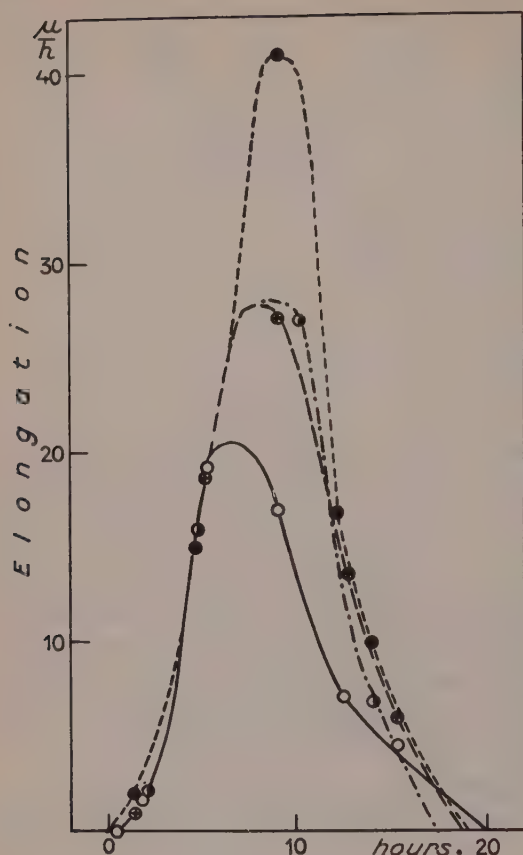


Fig. 8. The grand periods of elongation of cells in different concentration of P.C.I.B. — The graphs are superimposed so that phase I coincides. Thus the point »0 hours» is chosen for that purpose. Concentrations of P.C.I.B:

○ 0
 ● $3 \cdot 10^{-7}$ mol.
 ○ 10^{-6} "
 ● $3 \cdot 10^{-6}$ "

phase. The end result, increase or decrease in root length, depends upon the mutual balance between these two opposite effects. As to P.C.I.B it slows down the first phase, although this effect is rather slight at 10^{-6} mol, and quite obviously accelerates the second phase.

Summarizing, P.C.I.B has the following effects on the root growth. 1. It retards the first phase of the cell elongation. 2. It accelerates the second phase; this action dominates and results in the greatly increased gross length of the roots. 3. Partly but not wholly as a consequence hereof the root hairs disappear, and the development of lateral roots slows down. 4. In higher concentrations it retards the cell multiplication, perhaps a slightly specific effect, distinct from the real growth action.

In every respect, save for the last one, this is just the reverse of the actions of auxin on roots, and, in order to establish whether this is only a coincidence or not, experiments were carried out with combinations of auxin and P.C.I.B.

Tab. 2. *The interaction of P.C.I.B. and auxin on the cell length.* — Average of two series of experiments. Cell lengths given in μ after 2, 4, and 6 days respectively. Concentrations in Mol.

Treatment	Time days		
	2	4	6
Control	257 \pm 5	282 \pm 6	233 \pm 5
P.C.I.B. 3.10^{-6}	407 \pm 6	374 \pm 6	334 \pm 6
Auxin 10^{-8}	272 \pm 7	310 \pm 6	244 \pm 6
» + P.C.I.B.	361 \pm 7	324 \pm 7	280 \pm 6
Auxin 10^{-6}	82 \pm 3	267 \pm 5	184 \pm 5
» + P.C.I.B.	105 \pm 5	317 \pm 7	190 \pm 5

Combined effects of P.C.I.B. and auxin

The results of these experiments have been condensed into one table (table 2) giving only the cell lengths, which according to the statements above, are sufficient to express the action on the root growth. In accordance with previous results the effect of auxin on the cell multiplication is negligible or irregular.

Auxin was supplied in two concentrations. In 10^{-8} mol it may or may not initially retard the elongation, but with prolonged treatment it acts more or less accelerating. Thus, the action of auxin itself is slight, but it might increase when added to that of P.C.I.B. As a matter of fact when added to P.C.I.B. it causes a consistent, significant reduction in cell length as compared with the effect of P.C.I.B. alone.

In the concentration 10^{-6} mol auxin strongly retards the growth during the first interval, but shows a rapid recovery with irregular values for the cell length during the second and third intervals. This inhibition is likewise counteracted by P.C.I.B., so that after the second day the two compounds fairly well equilibrate each other in the tested concentrations.

It must be emphasized that the reverse actions of auxin and P.C.I.B. extends even to the adaptation. The roots adapt themselves to auxin as rapidly as they do to P.C.I.B. Another important point, definitely confirmed by this experiment, is that it is a question of adaptation, or change in sensitivity to the compounds. In previous experiments carried out with stagnant and at best frequently changed solutions this could be open to question, but with constantly flowing solutions around the roots there can be no doubt about the adaptation phenomenon.

Although all histological evidence speaks in favour of the conclusion that auxin and P.C.I.B. really act as antagonists, and the action of the latter

may be explained as a counter-action of auxin, it is necessary to see whether such an assumption conforms to the known facts of the mechanism of cell elongation, and the general conception of the auxin action.

Discussion of the auxin action on roots

The common picture of the auxin effect on roots is that it follows an optimum curve of the general type represented in figure 9. The broken co-ordinates are according to the picture chosen by Thimann (8). There are obvious flaws in this way of presenting the auxin action, however.

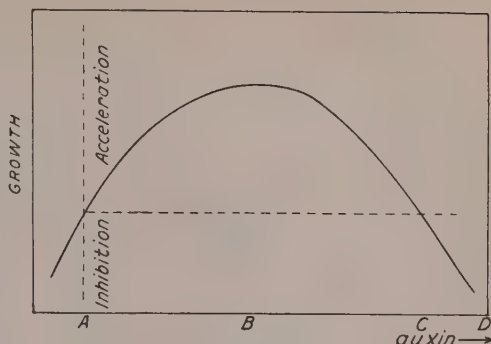
If we take auxin concentrations causing an accelerated growth they must lie between the points *A* and *C*. In every instance hitherto found — without addition of auxin, with auxin causing an acceleration from zero to about 40 per cent, or with no visible action at all — P.C.I.B. causes a very considerable increase in growth. Between points *A* and *B* this should imply a synergistic action of auxin and P.C.I.B., between *B* and *C* an antagonistic: the addition of P.C.I.B. equals a reduction in auxin activity.

If auxin retards growth, to the right of point *C*, P.C.I.B. obviously antagonizes auxin. Thus we have two possibilities, if the graph is theoretically correct: P.C.I.B. should — according to our present experience — act synergistically with auxin at low concentrations and antagonistically at higher levels; or, it has never been possible to realize the part *A—B*, but all experimental data on growth acceleration by auxin happen to fall between *B* and *C*, auxin and P.C.I.B. then counter-act each other under all circumstances. Further the tropistic responses of roots, as is well known, indicate that the normal state of roots is represented by a point somewhere between *B* and *C*, but then any positive effect of added auxin is inexplicable. This contradiction is, of course, generally realized but not seriously attended to in the literature.

On the basis of observations on the auxin effect on roots a theory was launched (2), which could account for this difficulty, and it is now pertinent to see whether it is applicable to the results with P.C.I.B.

According to this theory auxin has two different actions on the root cells; one during the start phase implying an acceleration of this part and consequently a tendency towards an increase in gross root growth, and another on the second part of the elongation, which is always impeded. Thus these actions are locally distinguished and partly independent of each other. The mechanism underlying the actions is of little interest in the present connexion. The second action dominates, however, so that auxin normally retards root growth. The roots can adapt themselves to some extent to an

Fig. 9. *The optimum curve of auxin action on root elongation.* — The broken co-ordinates according to the picture of Thimann (8). A denotes the normal condition without additional auxin, B optimum concentration, C inhibition compensating the acceleration, and D inhibition dominating.



addition of auxin, and then the first action will reveal itself in a growth acceleration. This is never very great, in the wheat material used for several years in our experiments the upper limit for a positive auxin action is an increase in cell length from about 250 to 300 μ . Nevertheless, this is by no means negligible and implies that the normal auxin content of roots cannot simply be described as overoptimal.

If we now try to compare this theory with the results on P.C.I.B., we can easily do so, because its action on the two growth phases is known. If added in low concentrations it retards somewhat the first phase and very much accelerates the second, in both respects counteracting the native auxin in the root. The latter effect dominates as usual.

The simplest way of illustrating the delay or retardation of the start phase has proved to be the measurement of the length of the meristem, before the elongation of the cells has started. Such average figures for control roots are 0.90 mm., P.C.I.B. $3 \cdot 10^{-6}$ 1.12 mm., auxin 10^{-6} 0.79 mm., and the combination of both 0.88 mm. Even at a gross inhibition of the elongation we find a significant shortening of the meristem, which means a more rapid start of the elongation.

That P.C.I.B. counteracts the inhibiting effect of auxin in this concentration on the second phase of the elongation is easily understood from the resulting cell lengths.

In this way the whole complex of the growth reaction can be explained by the following picture: 1. *The first phase of the elongation is favoured by auxin and retarded by P.C.I.B.* 2. *The second phase is retarded by auxin and promoted by P.C.I.B.* These two growth effects are, as a matter of fact, so locally separated and physiologically independent, that the graph of figure 9 has very little significance as a general picture of the auxin action. It is certainly impossible in view of the data presented, to indicate a point on such an optimum curve which should represent the condition of a given

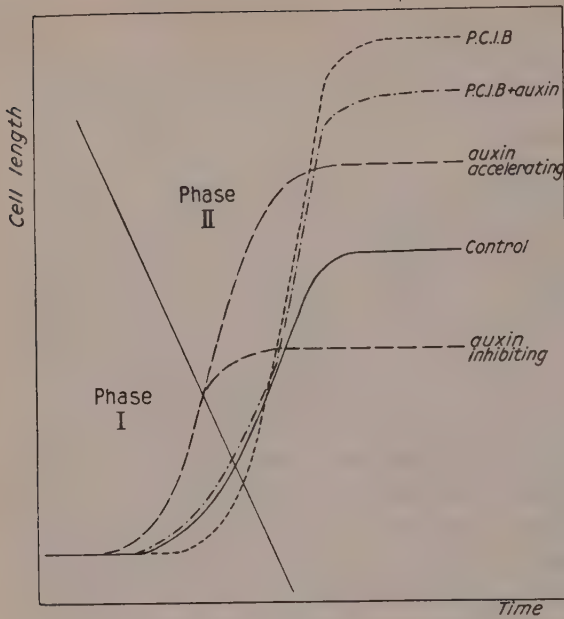


Fig. 10. *Schematic representation of cell elongation in auxin and P.C.I.B. solutions.* — The oblique line gives the approximate boundary between phase I with positive effect of auxin and negative or none of P.C.I.B., and phase II with negative or no effect of auxin and positive of P.C.I.B.

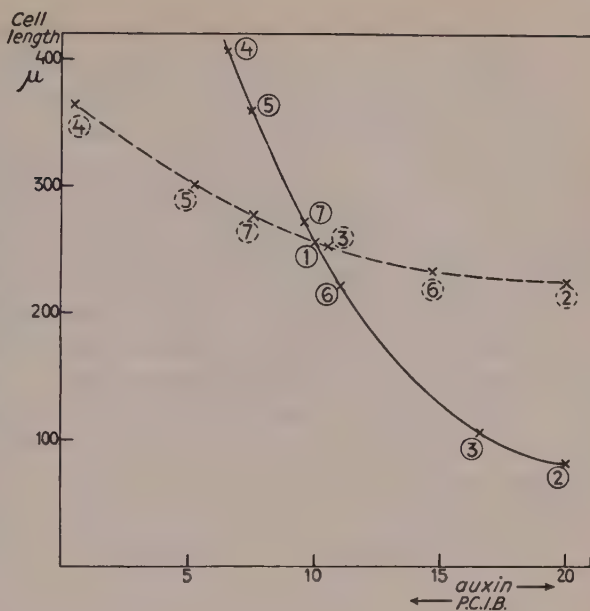
root, and to assume that the graph shows how the root should react on an addition of auxin, or a substance apparently acting as an anti-auxin like P.C.I.B. There is a positive and a negative auxin effect, but they have to be treated separately.

Figure 10 shows a slightly idealized picture of the growth curves of cells with additions of auxins, P.C.I.B., or both, according to previous (2) and the new results. The oblique line roughly separates phases I and II from each other. It shows how a promotion of phase I by auxin gives a final increase in length without a difference in phase II; it can never be very great, nor is it so in reality. The acceleration of phase II by P.C.I.B., on the contrary, can increase the cell length considerably, and changes in phase I are then of little consequence.

The crucial test should be, if it were possible to construct graphs as a substitute for figure 9, to show the actions on the two phases separately. For phase I this is at present impossible, because of the small effects, but with phase II an attempt has been made in figure 11.

These two graphs have been constructed from table 2 according to the following assumptions: the points should lie on a curve as smooth as possible, convex or concave against the abscissa. It was soon found impossible to construct an optimum curve from the figures. P.C.I.B. counteracts auxin in

Fig. 11. Graphs constructed to show the relation between auxin and cell elongation during phase II. — Solid line denotes the first to second day with strong inhibition by auxin, broken line recovery during third to sixth day. Constructed from the figures of table 2. 1=control, 2=auxin 10^{-6} , 3=auxin 10^{-6} + P.C.I.B, 4=P.C.I.B $3 \cdot 10^{-6}$, 5=auxin 10^{-8} + P.C.I.B, 6, theoretical figure for auxin 10^{-8} , 7=observed value for auxin 10^{-8} . Further explanations in the text.



such a manner that there is a fixed effect of each compound. In auxin 10^{-6} and P.C.I.B $3 \cdot 10^{-6}$ the effects on phase I can be neglected. Separate graphs have been drawn for interval 1 with strong auxin inhibition, and intervals 2 and 3 together with recovery.

The points on the graphs have been found in the following order and manner. (Figures refer to the numbers on the graph.) 1: control roots, placed at 10 units on the abscissa. 2: auxin 10^{-6} , placed arbitrarily at 20 units. 3: auxin 10^{-6} + P.C.I.B and 4: P.C.I.B only, both simultaneously placed at equal distances to the left of 2 and 1 respectively and giving a graph as smooth as possible; this can only be done if the curve is convex against the abscissa; otherwise it must contain one or more points of flexion. 5: auxin 10^{-8} + P.C.I.B placed on this graph. 6: found to the right of 5, and on the same distance as that between 1 and 4, respectively 2 and 3; this distance represents the action of P.C.I.B relative to auxin. Point 6 should now represent auxin 10^{-8} ; as a matter of fact, the experimental figures (point 7) are found much to the left of 6. The difference is supposed to depend upon the favourable action of auxin on the first phase, which cannot be neglected here. It is interesting to note that the theoretical point 6 is found to the right of 1, indicating an inhibition, which should be expected if the graph only represents phase II. The difference between 7 and 6, finally, showing the positive action of auxin, amounts to 43 and 49 μ in the two cases respectively. The actual top figures for positive auxin actions

in this material are 33, 37, and 51 μ increase in cell length. In view of the many approximations in the construction the agreement is rather surprising.

It is further striking, that the same distance is obtained between 6 and 7 for both intervals of time, which means that the change in sensitivity revealing itself as the adaptation, only occurs in phase II, but not phase I. This is just what must be expected to take place, or the change from inhibition to acceleration of the root growth could not be explained by the theory. In every respect the graphs in figure 11 fulfil the expectations.

The whole computation seems to indicate that the action curves for auxin on the second phase of elongation have the general shape of the graphs in figure 11. They are not optimum curves. On the contrary, they have the form to be expected if auxin has only an inhibitory effect, and this effect decreases relatively at higher concentrations, resembling a rather common kind of a physiologic action curve.

The construction further shows, that it is possible to explain the action of P.C.I.B quantitatively as an antagonist of auxin.

The action of P.C.I.B

The action of P.C.I.B must be explained with due consideration to its structure. The compounds of the phenoxy group as yet mainly exhibit negative effects on root elongation, even if positive effects have been recorded (7, 9). These gain in interest in view of the present results.

As mentioned in the introduction a blocking off of the side chain from the ring decreases the toxicity. It may be that this has completely vanished in P.C.I.B, and there only remains an antagonistic action to auxin. That should logically imply that the other phenoxy compounds likewise counteract auxins, but that the result of this effect is covered by secondary inhibitory actions.

Such an inhibitory action is assumed by Goldacre (6) to be responsible for the effect of 2,4-D but, of course, it cannot explain the consistent and strong inhibition of root elongation by this compound. On the other hand Muir and coll. (7) record positive growth actions of a number of phenoxy compounds on *Avena coleoptiles*. They act similarly to auxin and have even the same relative activity. This observation does not tally with ours. Muir's compounds, among which are found phenylbutyric acid and p-chlorophenoxyacetic acid, promote coleoptile elongation in concentrations of 10^{-5} to 10^{-4} mol, but do not cause more than about 30 per cent acceleration. At present it seems most probable that this positive action and the one

found on roots with P.C.I.B have little in common. It is also surprising how different results are obtained with different test methods, Muir calls attention to the disagreement between their results and e.g., those of Avery and coll. (1). It is obvious that a more detailed study of what is happening in the standard growth tests is urgently needed.

As to P.C.I.B, it should be denoted as an ideal anti-auxin for roots, devoid of any harmful effects in itself. The nature of the antagonizing action is, of course, unknown, but it is quite probable that there is some simple blocking of auxin from its place of action. P.C.I.B is less active than auxin; according to figure 11 these activities during the first interval should stand in the ratio of about 1 : 10, and for the following period of time about 1 : 3. This would be in accordance with some such blocking action.

Summary

The effect of α -(p-chlorophenoxy)-isobutyric acid, P.C.I.B, on the root growth has been investigated under constant conditions in flowing nutrient solutions.

The acid has the following effects. In concentrations around 10^{-6} and 10^{-5} mol it delays the start of the cell elongation and accelerates the second phase of the elongation. The result is that around 10^{-6} mol the top figure for the rate of elongation is more than doubled, the cell length is increased by about 75 per cent and the root length equally much. On prolonged treatment the roots partly adapt themselves and the growth acceleration decreases to about 40 per cent. The rate of cell multiplication does not change significantly.

On both phases of the cell elongation P.C.I.B has an action opposite to that of β -indolylacetic acid. Experiments with combinations of P.C.I.B and auxin lend support to the view that the former acts as an anti-auxin, perhaps blocking the native auxin, but devoid of all harmful effects in itself.

The theories of auxin actions on roots are discussed and the inability of the general picture to account for both positive and negative elongation actions is emphasized. It is pointed out that all data are in conformity with the author's theory of two independent, locally separated auxin actions, one positive on the first phase of elongation, another negative on the second phase, and that the antagonizing action of P.C.I.B supports this view, but is inconsistent with others. An attempt has been made to derive the true action curve of auxin on the second phase exclusively. It shows a continuous inhibition over the whole range of concentration available to experiments.

References

1. Avery, G. S. Jr., Berger, J. & Shalucha, B.: Comparative activity of synthetic auxins and derivatives. — *Bot. Gaz.* 104:281. 1942.
2. Burström, H.: The influence of heteroauxin on cell growth and root development. — *Ann. Agr. Coll. Sweden* 10:209. 1942.
3. — Studies on growth and metabolism of roots. I. The action of n-diamylacetic acid on root elongation. — *Phys. Plant.* 2:197. 1949.
4. — Studies on growth and metabolism of roots. III. The adaptation against growth inhibition by aliphatic C₁₂ and C₁₁ acids. — *Phys. Plant.* 3:175. 1950.
5. Erdtman, H. & Nilsson, G.: Synthetic plant hormones. I. Sulphur analogues of some phenoxy acetic acids. — *Acta Chem. Scand.* 3:899. 1949.
6. Goldacre, P. L.: On the mechanism of action of 2,4-dichlorophenoxyacetic acid. — *Austr. J. Sci. Res. ser. B*, 2:154. 1949.
7. Muir, R. M., Hansch, C. H. & Gallup, A. H.: Growth regulation by organic compounds. — *Plant Phys.* 24:359. 1949.
8. Thimann, K. V.: Plant hormones. — *The Hormones* 1:5. 1948.
9. Wilske, C. & Burström, H.: The growth-inhibiting action of thiophenoxy acetic acids. — *Phys. Plant.* 3:58. 1950.

Growth Factor Requirements of Isolated Wheat Roots

(A Preliminary Report)

By

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It has been shown in previous papers (1 and 2) that excised roots of barley and oats hitherto have failed to grow infinitely on account of a lacking, unknown factor affecting the meristem cell divisions. Moreover, since excised roots of other monocotyledons cease to grow after excising, there was reason for assuming that the meristem-activating factor was common for all monocotyledonous plants. Experiments with cultivating excised wheat roots, however, have proved that these roots grow more readily than roots of barley and oats, even in the same medium with the same additions of vitamins. Thus, these results indicate that the growth requirements of the roots of different species are very specific and cannot be directly transferred from one plant to another.

This paper contains some preliminary results of an investigation in progress of the growth of excised wheat roots, especially of the requirements of vitamins.

Methods

As experimental material roots of wheat (Weibull's »Eroica») have been used. The methods employed have been largely those already described (1 and 2). The wheat seeds, pretreated by soaking in a 0.1 per cent sublimate-formaldehyde solution, were sterilized by a treatment in a calcium hypochlorite solution only for 8 hours. The basic medium used has been that composed by Burström (1), containing 1/20 mol glucose, and it was sterilized by autoclaving for 20 min. at 110° C. Erlenmeyer flasks with 20 ml.

of solution have been used as culture vessels, and the growth took place in darkness. Each experiment included 5 parallel cultures with 4 roots. Although every root generally gave several laterals, only the main root has been measured for the growth studies.

Results

By a comparison of the growth of isolated roots of barley, oats and wheat in the usual medium at different temperatures, it was found that the wheat roots were diverging and had relatively great increases in length. This result has been the cause of the continued studies. A survey of the results of this experiment are given in table 1.

The growth increment per day of the wheat roots is on the average two to four times that of barley and four times that of oats. Further, there is an evident optimal temperature for the wheat roots at 27—28° C. It has, however, not yet been exactly localized. White (9) also found the same temperature optimum for wheat. On the contrary, the roots of barley and oats are not especially affected by the changes in temperature, which must be due to a growth limiting factor. A microscopical examination of the wheat root tips revealed that these had good meristem residues and, consequently,

Table 1. *The total lengths and growth per day in mm. of isolated roots of wheat, barley and oats cultivated for 10 days in darkness at different temperatures.*

Species	Temp. °C	20°	22°	25°	30°
Wheat	Total length	105	120	172	139
	Growth per day...	9.5	11.0	16.1	12.8
Barley	Total length	49	43	47	41
	Growth per day...	3.9	3.3	3.7	3.1
Oats	Total length	32	28	36	41
	Growth per day...	2.2	1.8	2.6	3.1

Table 2. *Total root length, growth per day, root diameter, meristem length and fresh weight of isolated roots of wheat cultivated for 12 days at 20° C in darkness.*

Time in days	0	1	2	3	4	5	6	7	8	9	12
Root length mm.	10	21	31	40	59	62	77	80	94	91	126
Growth per day mm.	—	11.0	10.5	10.0	9.7	8.4	11.1	10.0	10.5	9.0	9.6
Root diameter mm. ...	0.50	0.44	0.41	0.44	0.40	0.47	0.37	0.35	0.37	0.33	0.33
Meristem length mm.	1.20	1.05	1.07	1.04	1.12	0.83	1.03	0.89	1.04	0.63	0.46
Fresh weight per 20 roots gm.	0.05	0.08	0.12	0.14	0.17	0.18	0.19	0.29	0.29	0.26	0.56

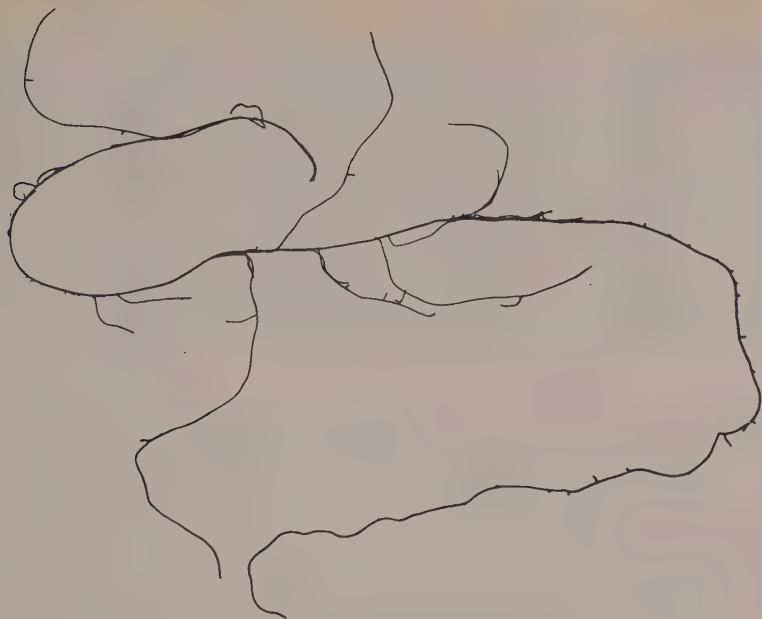


Fig. 1. *An excised wheat root cultivated for 24 days at 25° C in darkness together with 3 others in 20 ml. media. The main root was 48 cm. long. About 1/2 natural size.*

they should have been capable of further elongation. No studies on the meristem differences of the roots cultivated at different temperatures have hitherto been performed. In the root tips of barley and oats, as usual, only very few meristem cells were found. As the wheat roots had grown better and in spite of this had long meristematic parts, it is evident that the cell divisions must have continued after the excising of the root tip.

In order to study more closely the growth course, an experiment was performed in which the growth was studied every day for 12 days. It was started with 50 cultures, 5 of which were controlled every day. The results are given in table 2.

As is seen from the values of root length and growth per day, the isolated wheat roots elongate at the same velocity during the experimental period. Thus, their growth differs from that of excised barley and oat roots (1), the growth velocity of which soon diminishes and the root elongation ceases. A study of the values of root diameter and meristem length, however, shows that both are diminishing during the culture period. In another experiment the roots had only small meristems after 25 days of culture when kept at 20° C, but at 25° C the growth was still going on after the same length of time. The medium was not changed. Figure 1 represents one of these roots.

Table 3. *Total root length, growth per day, root diameter, meristem length (mm.) and number of laterals of isolated wheat roots cultivated at 20° C in darkness in solutions with different combinations of vitamins. The conc. are given in mg. per litre.*
(Pyr. = pyridoxine, Nic. = nicotinic acid.)

Vitamins	B ₁ 0.5 Pyr. 0.5 Nic. 0.5	Pyr. 0.5 Nic. 0.5	B ₁ 0.1 Nic. 0.5	B ₁ 0.1 Pyr. 0.5	B ₁ 0.1	Pyr. 0.5	Nic. 0.5
Root length	105	101	66	101	62	97	62
Growth per day	9.5	9.1	5.6	9.1	5.2	8.7	5.2
Root diameter		0.48	0.41	0.33	0.39	0.37	0.27
Meristem length		0.70	0.42	0.33	0.39	0.37	0.27
Number of laterals		4.0	5.9	6.5	6.5	1.7	7.2

Even if the nutrient medium employed does not fulfil the requirements necessary for infinite growth of wheat roots, these, however, are able to elongate considerably more than the roots of barley and oats in the same medium.

In the above mentioned experiments the nutrient solutions contained the following vitamins: thiamin 0.1 mg., pyridoxine (pyridoxine hydrochloride, Merck & Co. Inc., Rahway, N. J., U.S.A.) 0.5 mg. and nicotinic acid 0.5 mg. per litre. The results of experiments with the aim of studying the requirements of these substances are given in table 3 and figures 2 and 3.

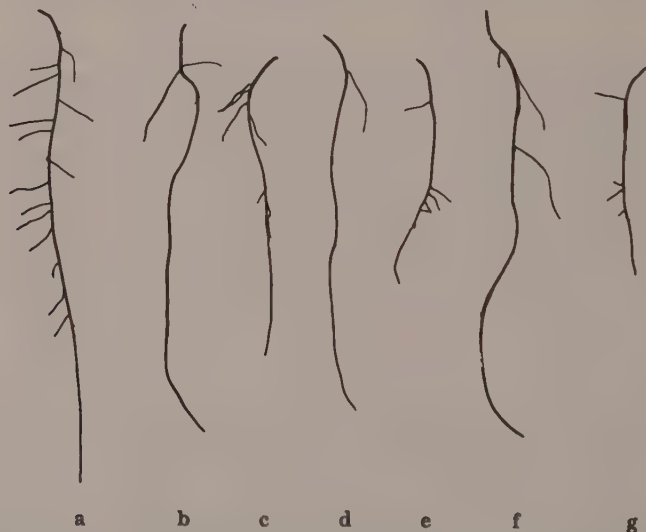


Fig. 2. *Representative excised wheat root cultivated for 10 days in darkness at 20° C in media with different combinations of vitamins. a) with B₁, pyridoxine and nicotinic acid, b) pyridoxine and nicotinic acid, c) B₁ and nicotinic acid, d) B₁ and pyridoxine, e) B₁, f) pyridoxine and g) nicotinic acid. (The concentrations are given in table 3.) About 1/2 natural size.*

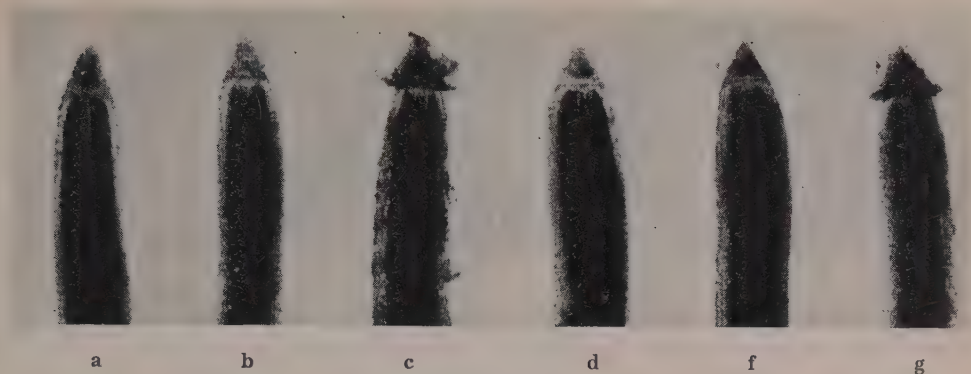


Fig. 3. Photomicrographs of the root tips of table 3 and figure 2. e) is omitted. Enl. 28 \times .

Both from the table and the pictures it is evident, that *pyridoxine is a decisive factor for the growth of wheat roots*. The total length diminishes, if the substance is eliminated and the number of laterals increases. The meristem length is greater when pyridoxine is supplied, and the same thing is true of the root diameter. The photomicrographs clearly show these facts. Unfortunately, there is no picture of a wheat root from a medium containing only thiamin, but such roots had very short meristems and many lateral initials. As is seen from c) and g) in figure 3 the roots from media lacking pyridoxine had the same appearance as the roots of barley and oats (see 1, p. 377). The meristem cells had elongated and no new cell divisions had taken place. A common phenomenon in the wheat root cultures is that long roots have long meristems, but short roots have short ones. This fact indicates that the limiting factor for growth mainly cell divisions and not cell elongation.

The results of an experiment on the determination of the optimal concentration of pyridoxine are given in table 4. The longest roots are found

Table 4. Total root length, growth per day, root diameter and meristem length (given in mm.) of isolated wheat roots cultivated for 30 days at 23° C in darkness in solutions with varied concentrations of pyridoxine hydrochloride. 0.1 mg. thiamin and 0.5 mg. per litre nicotinic acid are added in all cases.

Conc. of pyridoxine mg. per litre	0	0.1	0.3	0.5	1.0	5.0
Root length	71	170	239	327	356	269
Growth per day	2.0	5.3	7.6	10.6	11.5	8.6
Root diameter	0—0.05	0.7	0.7	0.7	0.7	0.7
Meristem length	0.33	0.41	0.41	0.37	0.39	0.36

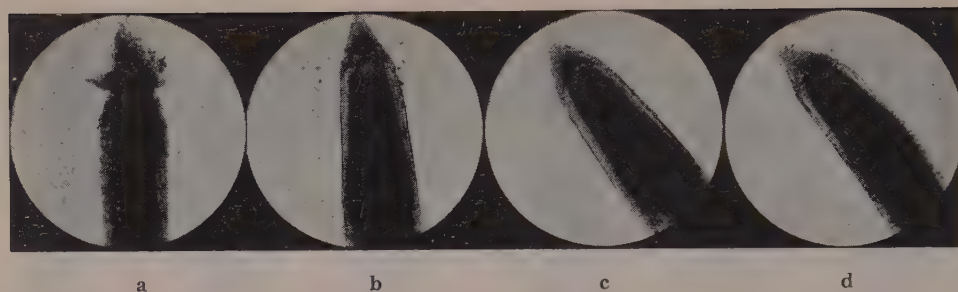


Fig. 4. Photomicrographs of the tips of excised wheat roots cultivated for 30 days at 23° C in darkness in media with varied concentrations of pyridoxine.

a) 0, b) 0.1, c) 0.3 and d) 0.5 mg. per litre. Enl. 27 \times .

in the medium with 1.0 mg. pyridoxine per litre, but the difference between 0.5 and 1.0 is relatively small. The values correspond to 10 and 20 γ per culture vessel with 20 ml. of solution. As regards the meristem length and root diameter there are no differences between varying concentrations of pyridoxine. The main difference is found between with and without pyridoxine. This is also apparent from the photomicrographs in figure 4. In the solution lacking pyridoxine cell divisions have ceased a long time before the control of the experiment, but in all other concentrations the appearance of the roots was the same. In 5.0 mg. per litre, however, the meristem cells were more vacuolated than in other concentrations, so this concentration seems to be supraoptimal.

Discussion

Pyridoxine has earlier been used e.g., for isolated tomato roots, but the results of different investigators are not in agreement. Thus Bonner (3), Bonner and Devirian (4), Day (5), and Robbins and Schmidt (6 and 7) all obtained growth promoting effects. White (10), however, stated that pyridoxine is without effect on tomato roots. The contradictions partly depend upon different tomato root material, which is also found by Whaley and Long (8). Thus even roots of the same »species» have different pyridoxine requirements and so it is not surprising that the author has found that the substance is without any effect on roots of barley and oats (1). The present paper, however, shows that pyridoxine is necessary for the growth of isolated wheat roots and produces cell divisions in the meristems.

The results hitherto obtained in wheat root cultures have not proved, however, that it is possible to cultivate these roots infinitely, for a few experiments performed have shown that at 20° C the roots of the second passage

became hair-thin and stopped growing. The growth conditions at 25° C, however, indicate that it can be possible if the climate is optimal.

According to the general studies on the effects of pyridoxine in the organism, it functions in the metabolism of amino acids and studies on the protein synthesis in the roots are in progress. It seems possible that this process is disturbed in the isolated roots of barley and oats, as the increase in dry substance of these roots is very minute.

Summary

Isolated roots of wheat have been cultivated in media according to methods earlier described and already used for cultures of roots of barley and oats. The wheat roots grow much better than these species and it has been shown that of the vitamins thiamin, pyridoxine and niacin, pyridoxine alone has a decisive effect on the growth by promoting the meristem cell divisions. Optimal growth has been obtained in pyridoxine concentrations from 0.5 to 1.0 mg. per litre at 27—28° C.

References

1. Almqvist, A.: Studies on the growth of isolated roots of barley and oats. — *Physiol. Plant.* 2: 372. 1949.
2. — Further studies on the growth of isolated roots of barley and oats. — *Ibid.* 3: 205. 1950.
3. Bonner, J.: On the growth factor requirements of isolated roots. — *Am. J. Bot.* 27: 692. 1940.
4. — and Devirian, J. S.: Growth factor requirements of four species of isolated roots. — *Ibid.* 26: 661. 1939.
5. Day, D.: Vitamin B₆ and growth of excised tomato roots in agar culture. — *Science.* 94: 468. 1941.
6. Robbins, W. and Schmidt, M. B.: Further experiments on excised tomato roots. — *Am. J. Bot.* 26: 149. 1939.
7. — and —: Vitamin B₆, a growth substance for excised tomato roots. — *Proc. Nat. Acad. Sci., Wash.* 25: 1. 1939.
8. Whaley, G. and Long, A.: The behavior of excised roots of heterotic hybrids and their inbred parents in culture. — *Bull. Torr. Bot. Club.* 71: 267. 1944.
9. White, Ph. R.: Influence of some environmental conditions on the growth of excised root tips of wheat seedlings in liquid media. — *Plant Phys.* 7: 613. 1932.
10. — Vitamin B₆, nicotinic acid, pyridine, glycine and thiamin in the nutrition of excised tomato roots. — *Am. J. Bot.* 27: 811. 1940.

Growth Studies in Woody Species II. Effect of Day-Length on Shoot-Growth in *Pinus silvestris* after the First Year

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In the first paper of this series (Wareing, 1950 b) photoperiodic effects in first-year seedlings of *Pinus silvestris* were described. The present paper deals with the effect of day-length upon the growth of older seedlings and is a further contribution to a general study of growth-periodicity in *P. silvestris*, which formed the subject of a thesis accepted for the degree of Ph. D. in the University of London (Wareing, 1950 a). A preliminary report of this work has already appeared (Wareing, 1949).

The growth of the annual shoot in *P. silvestris* in the second and later years differs from that of first-year seedlings in that it is limited to extension of internodes already laid down in the resting-bud (Wight, 1933), and when this has been completed (in May or early June) there is no further period of extension-growth by the continued formation of new initials in the apical region, as occurs in many woody species (e.g. *Robinia pseudacacia*). Thus, after the first year of growth the number of nodes formed on the annual shoot of *P. silvestris* is predetermined by the number of scale leaves formed in the terminal resting-bud in the previous year. It is clear, therefore, that the day-length conditions prevailing during the period of extension of the annual shoot in *P. silvestris* cannot affect the number of nodes developed. The following experiments were, however, carried out to determine whether any other photoperiodic effects are observable in older seedlings. The possibility of such effects had already been suggested (Bünning, 1939, Langlet, 1942—3) from observations on the results of 'provenance' experiments with *P. silvestris*, but direct experimental demonstration was still lacking.

Experimental

Effect of day-length on extension of the annual shoot

Experiment 1

Methods. Twelve pots, each containing 3—4 two-year old plants which had previously been grown in the open under natural day-length conditions, were brought indoors early in January. After about one month the buds showed signs of breaking dormancy, and the plants were then divided into two equal series of 6 pots each, and exposed to day-length conditions as follows. Both series were exposed to natural daylight from 9 a.m. to 5 p.m., and each series was then transferred to one of two identical light-proof covers consisting of a wooden framework covered with black cloth. Each cover consisted of a lower section (approximately 50 cms. cube) and an upper section which was separated from the lower by an asbestos sheet. A 75-watt filament-lamp was housed in the upper section of each cover, above an aperture (8 cms. square) in the asbestos sheet. This aperture was covered with a sheet of glass, which in one cover was coated with an opaque layer of black unvarnished paint. A water-screen consisting of a large crystallizing dish containing a depth of 5 cms. of water was interposed between the lamp and the aperture in both covers. Thus, the only difference between the two covers was that in one case the glass over the aperture was opaque, so that heating effects from the lamps were common to both covers, and tests showed that there were in fact no detectable differences in temperature between the two covers. The lamps were switched on at 5 p.m. and off at 11 p.m., so that in one cover the plants received 6 hours' additional artificial illumination (at an intensity of 100—120 lux) to give a total daily photoperiod of 14 hours, while in the case of the plants in the other cover no artificial illumination was received and they were thus exposed to a daily photoperiod of 8 hours.

The temperature of the greenhouse was thermostatically controlled at 15° C, but in bright weather tended to rise above this.

Results. Growth of the young shoots, following the breaking of the buds, was rapid and was completed in 6—8 weeks. As growth progressed, differences became apparent between the two series, the extension of the new shoots of the 'short-day' series being appreciably reduced as compared with those under 'long days'. When extension of the shoots had ceased the mean length of the new growth of the 'leaders', and the mean length of the leaves at the mid-point of the new shoot, were determined for each series, the data being summarised in Table 1. Representative plants of the two series are shown in Figure 1. It is seen that there is a difference of 1.8 cms. between the mean length of the new shoots of the two series, and this difference is fully significant (for 't' test, $P < 0.01$). Since the mean number of internodes must have been the same in both series, this difference in total shoot-length must reflect a difference in mean internode length between the two series. Now when it is considered that the additional period of illumination received

Table 1. *Effect of day-length on shoot-extension and leaf-growth (Experiment 1).*

Photoperiod	No. of plants	Mean length of new shoot (cm)	Mean length of leaves (cms)
8 hours	20	5.86 ± 0.43^1	2.20 ± 0.09
14 hours	23	7.65 ± 0.53	4.29 ± 0.22

¹ In this and other tables the Mean \pm Standard Error are quoted.

by the 'long day' plants was at 100–120 lux only, it is clear that this is a photoperiodic effect, and that extension of the internodes is reduced under short days, just as in the first year of growth. The difference in leaf-length is also clearly significant and is dealt with below.

Experiment 2

In order to obtain confirmation of the results of Experiment 1, and to obtain more detailed information on the effects observed, the following experiment was carried out.

Methods. In early April, sixteen pots of two-year old plants were divided into two series. One series ('long day') was exposed to natural day-length conditions (ranging from approximately 15-hours to 17-hour photoperiods) and the other series ('short-day') was exposed to a photoperiod of 10-hours, by covering with a light-proof cover from 5 p.m. to 7 a.m. Both series were grown in the open.

Results. (a) *Shoot extension.* When extension of the shoots had ceased (3rd June), the mean length of the new growth was determined for the 'leaders' of both series, the data being contained in Table 2. It is seen that the mean length of the shoots under short days is again significantly less than under natural day-lengths, thus confirming the results of Experiment 1.

(b) *Apical dominance.* In order to determine whether the length of the photoperiod affected the *relative* growth of the 'leaders' and lateral shoots, the mean length of the lateral shoots arising at the base of the new 'leader' growth was determined (Table 2). From the data so obtained the ratio: mean length of leaders/mean length of laterals, was determined for each series. The values for this ratio were 1.65 and 1.60 for the 'long day' and 'short day' series respectively. The close agreement between these two values indicates that the laterals of the 'short day' series showed proportionately the same reduced extension as the leaders, and that apical dominance was not appreciably affected by the length of the photoperiod.

(c) *Leaf-growth.* It was observed that in early June, when extension of the shoots had effectively ceased, the leaves of both series of plants were still very short (1–2 cms. in length), and indeed the presence of a soft



Fig. 1. Third-year plants of *P. silvestris* grown under (A) 8-hour day, and (B) 14-hour day, during the third season of growth.

succulent region at the base of the leaf indicated that growth by activity of the basal meristem was still continuing. Continuous observations were therefore carried out on the further growth of the leaves. By mid-July the basal portions of the leaves of the 'short day' series had become tough and fully differentiated, and it was evident that leaf-growth in this series had then ceased. Active leaf-growth in the 'natural day' series continued until early September, when the same changes occurring at the leaf base indicated that leaf-growth had ceased.

Confirmatory evidence that the presence of a succulent, light-coloured region at the leaf-base indicates that leaf-growth is still active was obtained by making a mark on the leaf with Indian ink at the point where the leaf-base emerges from the 'sheath'. In this way any growth of the leaf at the base was easily observed, and it was found that in the 'natural day' series there was still active extension of the leaves in early August, but by the end of August growth was markedly diminished and simultaneously with the visible changes occurring in the basal region, further growth ceased.

Table 2. Effect of day-length on shoot-extension and leaf-growth (Experiment 2).

Photoperiod	No. of plants	Mean length of new 'leader' growth (cms)	Mean length of new lateral growth (cms)	Leader growth/lateral growth	Mean leaf length (cms)
10 hours	30	7.23 ± 0.35	4.52	1.60	3.0 ± 0.15
Natural day-length ...	40	9.90 ± 0.33	5.97	1.65	7.0 ± 0.25

Thus, the observations of Tolsky (1913) that leaf-growth of *P. silvestris* in southern Russia continues until August, long after shoot-extension has ceased, was confirmed. This conclusion was also confirmed by observations carried out on mature trees growing under naturalised conditions in Southern England.

The marked difference in final leaf-length between the 'long-day' and 'short-day' plants (Table 2) in the present experiment is thus to be ascribed partly to the reduced growing period of the leaves under 'short day' conditions.

Further Observations on Leaf-growth

Experiment 3

The results of the foregoing experiment clearly indicate that day-length conditions affect the duration of growth of the leaves in *P. silvestris*. It is not clear from the facts so far described, however, whether the reduced duration of growth of the leaves under 'short days' is a direct effect, or is a secondary one resulting from differences in extension of the stem. In order to obtain direct information on this point, the following further experiment was carried out.

Methods. Second-year plants in pots were allowed to grow under natural day-length conditions during the spring, until elongation of the shoot had ceased. Early in June they were divided into three series of six pots each, and the plants of each series were removed from the pots and planted together in a box of soil. All series of plants thus commenced with stems of equal length. From early June the three series were exposed to 12-hour, 14-hour and natural day-length conditions respectively. Observations were made as to the duration of leaf-growth, by removing single leaves and examining the basal meristem (as described in Experiment 2). Using this criterion, the date on which leaf-growth ceased in each series was determined. After leaf-growth had ceased in all plants, the mean leaf-length of each series was determined by measuring the lengths of 3 leaves from each plant, taken at random. The data are summarised in Table 3.

Results. It is seen that the duration of growth and final leaf-lengths increased with the length of the photoperiod. These differences in leaf-length are all statistically significant (for 't' test, $P < 0.001$). Thus direct photoperiodic control of leaf-growth may be regarded as established.

Experiment 4

Since, under natural day-length conditions, leaf-growth was observed to cease at about the end of August in trees of various ages and under a variety of cultural conditions (both in pots and under field conditions) the question arises as to whether cessation of leaf growth under natural conditions is

Table 3. *Effect of day-length on leaf-growth.*

Series	Photoperiod	No. of plants	Mean leaf-length (cms)	Cessation of leaf-growth
A	12	53	5.18 ± 0.17	end July
B	14	54	6.20 ± 0.15	early Aug.
C	'Normal'	56	8.68 ± 0.18	end Aug.

controlled by the falling natural day-length in August. In order to obtain information on this point two experiments were carried out in successive years. On 8th August, 1948, six pots of second-year seedlings were divided into two equal series (containing a total of 12 plants in each). All plants had previously been grown under natural day-length conditions, but from 8th August one series was allowed to remain under natural photoperiods, whereas the other received additional artificial illumination from dusk until 9 p.m., giving a total photoperiod of 16—17 hours' duration. In both series of plants leaf-growth ceased at the end of August. In a similar experiment carried out in 1949, 12 pots, each containing 4—5 second-year plants were selected on 15th August, when leaf-growth was still active. They were divided into two equal series and one was given supplementary illumination at an intensity of 150 lux to extend the natural photoperiod to 16 hours. The natural photoperiod at the commencement of the experiment was about $15\frac{1}{2}$ hours. Leaf-growth continued in both series until the first week in September, when the natural photoperiod is about $14\frac{1}{2}$ hours, but by that date it had ceased in both series. In both these experiments, therefore, additional illumination to extend the natural day-length to about 16 hours during the latter part of August failed to prolong the duration of leaf-growth as compared with that of plants grown under natural photoperiods, and there is, therefore, no evidence that in the experimental plants cessation of leaf-growth was controlled by a reduction in the natural length of day.

Location of Photoperiodic Perception in P. silvestris

It is well-known that the location of photoperiodic perception in herbaceous species is in the mature leaves (Cailachjan, 1936). It is of some interest, therefore, to know whether photoperiodic perception in older plants of *P. silvestris* occurs in the young extending shoot, or in the older leaves of the previous year's shoot. *Prima facie* considerations suggest the latter alternative, for in the growth of the new annual shoot extension occurs very rapidly and has been completed by the time the new leaves are little more than 1 cm. in length. In order to obtain information on this question

Table 4. *Effect of day-length and defoliation on shoot-extension.*

Treatment	Number of plants	Mean length of new shoots
Long day (1) Intact	27	12.18 \pm 0.62
» » (2) Defoliated	23	5.01 \pm 0.27
Short day (1) Intact	28	10.18 \pm 0.44
» » (2) Defoliated	19	4.82 \pm 0.45

a preliminary experiment was carried out, in which defoliation of the old leaves was effected, and the plants then exposed to two different photo-periods of 8 hours' and 14 hours' duration respectively. It was to be expected that such plants would make reduced growth, if only because of the absence of current assimilation, but it was hoped that sufficient growth would be made to permit detection of any effect due to the influence of different day-lengths conditions. In fact, however, the growth of the defoliated plants was very poor and no significant difference between the 'long day' and 'short day' series was detected. The results are shown in Table 4, which also includes, for comparison, data for two parallel series of plants in which the leaves were allowed to remain intact. In about 1/5th of the plants of both the defoliated series the buds failed to break dormancy and the plants died. Evidently the presence of the older leaves is necessary for normal breaking of the buds and extension of the shoots in *P. silvestris*.

Although new growth is made only with difficulty in defoliated plants, the results of Experiment 7 described below show that considerable, rapid, growth may be made even in complete darkness, provided the old leaves are left intact. This fact was made use of in the following experiment.

Experiment 5

Methods. Twelve pots of 2-year old seedlings were divided into two equal series, and the stems of all plants were individually wrapped in black cloth, tied at top and bottom, so that only the terminal buds were left exposed. In this way the old leaves were kept in continuous darkness, while the new shoots of one series were exposed to a 'long day' and of the other to a 'short day'. The lateral buds at the apex were removed from all plants, leaving a single terminal bud. Both series of plants were exposed to illumination at 4000—5000 lux from a battery of eight 80-watt fluorescent lamps suspended 25 cms. above them, for 6 hours daily. The plants of one series ('short day') were kept under a dark cover between the periods of illumination and were thus exposed to a daily photoperiod of 6 hours. The other series ('long day') received a further period of illumination at 250 lux from a 100-watt filament lamp for a further 10 hours, giving a 16-hour photoperiod, and 8 hours darkness. In order to eliminate any heating-effect during this period of low-intensity illumination, a water-screen consisting of a large

Table 5. *Data for Experiment 5.*

Series	Number of plants	Length of new shoots	
		On 9th day	At end of experiment
A	19	3.05 ± 0.20	4.31 ± 0.32
B	19	4.02 ± 0.31	6.67 ± 0.49

crystallizing-dish containing 4—5 cms. of water was placed between the lamps and the plants. There were thus no appreciable temperature differences between the plants under the dark cover and those receiving additional illumination. The room temperature was maintained nearly constant at 15° C, except during the period of illumination by the fluorescent lamps, when it rose to approximately 20° C.

The buds of both series of plants showed signs of breaking dormancy at the commencement of the experiment. The length of the expanding buds and of the new shoots was measured individually for each plant at intervals of 3 days throughout the experiment. The data are given in Table 5 and Figure 2.

Results. It is seen from Fig. 2 that the rate of extension of the shoots of the 'long day' series was greater than that of the 'short day' series from the commencement of the experiment, and that on the 9th day there was already a significant difference in shoot length between the two series (for 't' test, $P < 0.01$). At this time the young leaves of the new shoots had still not emerged from the basal sheath in the majority of plants, and in the remainder the tips of the leaves projected for only 1 mm. beyond the sheath.

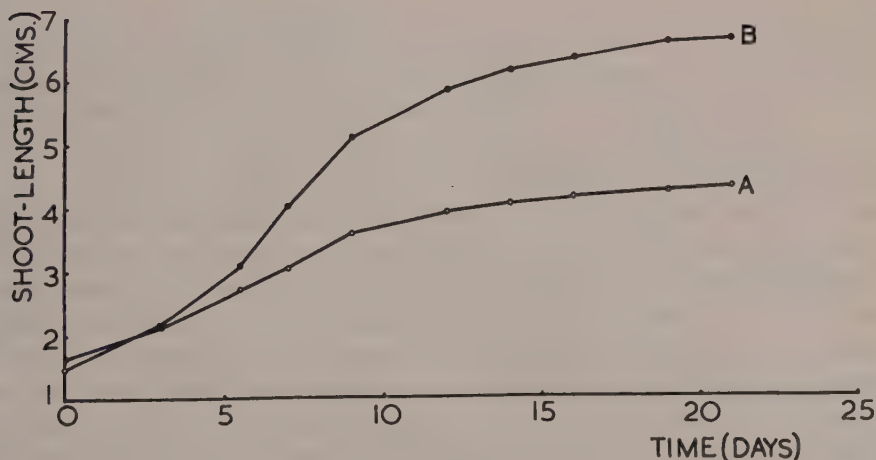


Fig. 2. Extension of the annual shoot under (A) 6-hour day and (B) 16-hour day. (Experiment 5.)

Table 6. *Data for Experiment 6.*

Series	Number of plants	Length of new shoot (cms)	Length of leaves at end of experiment (cms)
A	33	5.37 ± 0.27	1.27 ± 0.11
B	31	6.33 ± 0.34	2.15 ± 0.07
C	31	11.03 ± 0.44	2.66 ± 0.07

Thus a greater extension of the young shoots occurred under long day conditions even in the very early stages, at a time when the needles of the young shoots were still in a very rudimentary state. There seems no doubt, therefore, that the additional low-intensity illumination to which the plants of series B were exposed had a direct effect upon the internodes. This appears the more remarkable when it is considered that the additional illumination was directed from a lamp vertically above the plants, so that the incident light on the sides of the young shoots must have been considerably less than 250 lux.

The difference in rate of shoot extension between the two series was maintained for a further 14 days, when growth in both series had effectively ceased. At this date the uppermost internodes had not fully extended and it seems probable that growth ceased at this stage because of the exhaustion of food reserves, since the young leaves of the new shoots were still very short, and the capacity for assimilation of the plants must have been very low.

Experiment 6

The results of the previous experiment show that there is a direct photoperiodic effect upon the extending young shoots. The possibility still remains, however, that some effect may also be exerted through the agency of leaves of the previous season. In order to test this possibility it is necessary to maintain the young shoots under constant day-length conditions, and to expose the older leaves of two different series of plants to long and short photoperiods respectively. This, however, would involve the excessively laborious process of covering and uncovering the old leaves of a considerable number of individual plants each day. As an alternative, therefore, in the following experiment the old leaves of one series of plants were kept in continuous darkness (as in the previous experiment) while the new shoots were exposed to a 6-hour photoperiod, and the growth of these plants was compared with that of plants in which both the old leaves, and the new shoots, were exposed to a 6-hour photoperiod. If the day-length conditions to which the old leaves are exposed have any effect on the new shoot, it is

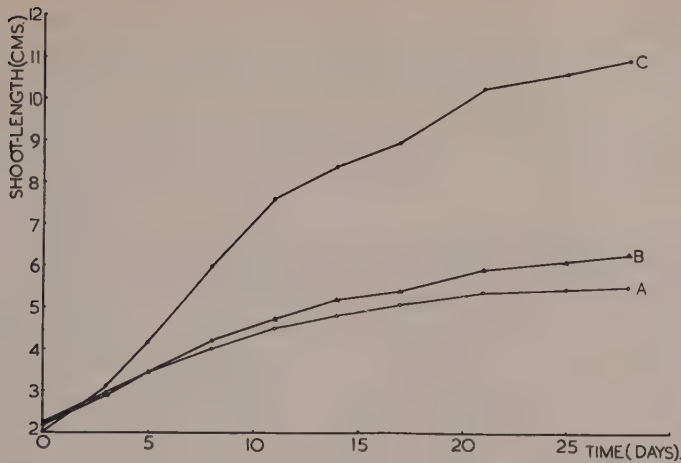


Fig. 3. Extension of the annual shoot, Experiment 6. (For details see text.)

to be expected that some difference should be observed between the two series under the conditions described.

Methods. Eighteen pots of 2-year old plants were brought into the laboratory on 3rd April, when breaking of the buds had commenced and were divided into 3 equal series. In one series (A) the stems of the plants were wrapped in black cloth to cover the older leaves, but in the other two series the leaves were allowed to remain uncovered. All series of plants were exposed to illumination from fluorescent lamps for 6 hours daily, as in the previous experiment. The plants of series A and also of a second series (B) were kept under a dark cover in the laboratory between the periods of illumination, while the third series (C) received a further period of illumination for 10 hours at 250 lux (under the conditions described for the long day plants of Experiment 5), giving a 16-hour photoperiod and 8 hours' darkness.

The length of the new shoot was measured individually for each plant in all series at intervals of 3 days, and the data are given in Table 6 and Figure 3.

Results. The arrangements of the experiment were such that the effects of continuous darkness (series A) and 'short days' (series B) on the old leaves could be compared. The third series of plants under 'long days' was introduced in order to verify that any photoperiodic effects were not limited by the low assimilation occurring under the conditions of the experiment. (This was already shown from the results of Experiment 5, but the present experiment actually preceded the latter.)

It is seen that there was very little difference between the growth of series A and B during the first 17 days of the experiment, the slightly reduced growth in series A over this period possibly being due to the

absence of current assimilation in these plants. Thus, during the first 17 days of the experiment covering of the leaves of the previous year's shoot had no appreciable effect on the growth of the current year as compared with that in plants of which the old leaves received 6 hours' light per day. After 17 days the difference between the two series became greater and this was seen particularly in the greater growth of the leaves on the new shoots of series B. The plants of both these series showed the marked reduction of internode extension already found to be associated with short photoperiods. On the other hand, the shoots of plants exposed to a 16-hour day showed a very much greater extension of the internodes, and an increased rate of extension was observable from the beginning of the experiment. Thus it was evident that the close agreement between the growth of series A and B was not due simply to the fact that in both series it was limited by the low rate of current assimilation, for greatly increased growth was obtained in series C with low-intensity illumination which could have little effect on assimilation. The conclusion may therefore be drawn that the day-length conditions to which the older leaves are exposed has very little, if any, influence on the growth of the new shoots. This matter is discussed further below.

Experiment 7. Effect of continuous darkness.

Since short days have a direct effect in reducing the extension of the internodes, it is of interest to know what is the effect of complete darkness on extension of the new shoots. In many species, of course, when the daily quantity of light received by the plants is reduced below a certain minimum, 'etiolation' effects become apparent, so that in continuous darkness there is actually *elongation* of the internodes as compared with those of plants grown in the light. These etiolation effects include certain disturbances in the morphogenesis of the leaves when the plants are kept in complete darkness, but the development of the leaves and internodes can be rendered more nearly 'normal' by exposure to quite small quantities of light (Priestley, 1925). In the following experiment, therefore, one series of plants was kept in continuous darkness, and another series was exposed to a daily alternation of 12 hours' low-intensity illumination at 150—200 lux and 12 hours' darkness, in case the first series should be too abnormal to afford comparison with plants grown in the light. Now it has been shown (Mann, 1940) that in herbaceous species a daily light period entirely at low intensity is ineffective for photoperiodic induction of flowering, and it was tentatively assumed therefore that in the present experiment the effect of such illumination would be without 'photoperiodic' effects on the extension of the shoot, but would reduce the etiolation effects resulting from complete darkness.

Table 7. *Effect of continuous darkness and low-intensity illumination on shoot-extension.*

Treatment	Number of plants	Mean length of new shoots
Continuous darkness	29	6.64 ± 0.50
12 hours' illumination + 12 hours dark	26	6.53 ± 0.51

Methods. Twelve pots of 2-year old plants were brought into the laboratory dark-room in early April, when signs of breaking of dormancy were apparent. Six of these pots of plants were then kept in continuous darkness and the remaining six were exposed to a daily alternation of 12 hours' illumination at 150—200 lux from a 100-watt lamp and 12 hours' darkness. Watering of the plants kept in complete darkness was carried out every 2—3 days, and during this operation the plants were exposed for a few seconds to a photographic safe-lamp for observation.

Results. Growth of the shoots was very rapid in both series of plants and had effectively ceased within 3 weeks. The final lengths of the new shoots are given in Table 7. It is seen that there was no significant difference in the lengths of the new growth of the two series. At this time, in both series, many of the internodes in the apical region remained unextended, and it therefore seems probable that further extension was limited owing to the exhaustion of some essential factor. Although the mean total lengths of the new shoots in the two series did not differ significantly, there was a difference in the distribution of growth, the length of the lower internodes being clearly greater in the case of the plants kept in complete darkness, and the number of extended internodes must therefore have been fewer. Moreover the new shoots of the plants maintained in darkness were colourless, whereas in those grown with a daily exposure to 12 hours' light, the stems and leaves were green. There were, however, no other observable morphological differences between the two series.

The chief point of interest of this simple experiment is that although 'short days' result in reduced extension of the shoot, active growth can still occur even in continuous darkness. This result is discussed below.

Since the plants of the present experiment were derived from the same stock as those used in the defoliation experiment described above, it is of interest to compare the rapid extension and greater total growth made by the plants of the present experiment with the very slow growth made by the defoliated plants.

Discussion

It has been demonstrated in the experiments described above that, although after the first year the duration of growth is pre-determined by the number

of node-initials laid down in the bud, nevertheless the extension of the internodes is affected by day-length conditions, short days producing reduced internode extension. In this respect the effects observed in *P. silvestris* agree with the general observation that short days produce shortened internodes, and a tendency to a 'rosette' habit, in both long-day and short-day herbaceous species (Garner, 1936). This effect on internode extension appears to be a direct one, and is not a secondary one arising from a photoperiodic 'perception' in the leaves, as is generally held to be the case in herbaceous species. The increased extension of the hypocotyl of seedlings of *P. silvestris* observed by Karschon (1949) appears to be of a similar nature, since the cotyledons are still largely enclosed in the testa during the period of extension of the hypocotyl. A direct response of the stem to photoperiodic stimulus has been reported by Dostal (1944) for the rhizomes of *Circaea intermedia*. Similarly Harder and Westphal (1944) observed that some inhibition of flowering occurs when the defoliated stems of the short-day species *Kalanchoë Blossfeldiana* are illuminated. In these last two cases, however, it is fully-differentiated stem tissue which is the site of perception, whereas in *P. silvestris* the effect appears to be directly upon meristematic tissue which is in the process of extension.

It might be suggested that the greater extension of the internodes under 'long days' as against 'short days' is due to the greater production of some substance, formed by a photocatalytic reaction, under 'long day' conditions. On this basis, extension should be at a minimum in continuous darkness. In fact, however, it is found that extension can occur rapidly even in continuous darkness (although growth ceases before the upper internodes have extended). This observation supports rather the suggestion put forward in connection with the phenomena observed in first-year seedlings (Wareing, 1950 b) viz. that the reduced internode-length under short-days is due to the *inhibitory* effect of long dark periods. It is well-established that the effect of the dark period in photoperiodism is dependent upon there being a preceding period of illumination (Mann, 1940) and hence the capacity for extension even in continuous darkness in *P. silvestris* may be due to the fact that under such conditions the inhibitory action of the dark period is lacking, because of the absence of accompanying periods of illumination. It is, of course, possible that the mechanism of extension of etiolated stems in continuous darkness is different from that of shoots in the light, but even if these etiolation effects are reduced by low-intensity illumination (which is generally assumed to have no photoperiodic effect, except when used to supplement a period of illumination at a higher intensity [see Gregory, 1948]), extension is still possible.

It has been shown that although the day-length conditions to which the

leaves of the previous years' shoot are exposed have little or no effect on the extension of the current year's shoot, nevertheless the older leaves apparently contain some material in the absence of which the breaking of the buds and extension of the shoots occurs only with great difficulty. In view of the well-known importance of auxin in shoot extension it seems possible that a reserve of auxin or auxin precursor is normally present in the old leaves, but the possibility that other factors are also involved is by no means excluded. It seems clear that the function of the leaves in this connection is not simply that of current photosynthesis, since active extension of the shoot can occur even in the dark, provided that the old leaves are left intact.

It has been shown that the duration of leaf-growth is affected by the day-length conditions, but attempts to prolong the duration of leaf-growth by providing supplementary illumination during August were unsuccessful. Now, from the results of Experiment 3 it is clear that under a given *constant* photoperiod a certain mean 'maximum leaf-length' can be attained and then the basal meristem becomes dormant. It would appear, therefore, that under natural day-length conditions the 'maximum leaf-length' for a photoperiod of 15—16 hours has already been nearly attained by mid-August and that prolonging the natural day-length by a relatively short period does not appreciably affect the duration and final amount of leaf-growth. Nevertheless, although there is no evidence of direct photoperiodic control of the duration of leaf-growth by natural day-length changes in Southern England, these photoperiodic effects are likely to be important in provenance-experiments where races from localities of widely different latitude (and hence of mean day-length) are involved.

The work forming the subject of the present paper was carried out whilst the author was a number of the staff of Bedford College, London. He wishes to re-affirm the acknowledgements already made in the first paper of this series.

Summary

1. After the first year of growth in *P. silvestris*, the number of nodes is pre-determined by the number of initials laid down in the buds in the previous year, so that the duration of growth cannot be affected by day-length conditions. Nevertheless the extension of the internodes is affected, being reduced under short-day conditions.

2. It is found that the length of the needles is reduced under short-day conditions and this effect is partly due to the fact that the cessation of leaf-growth occurs sooner under short-day conditions. There is no evidence

of direct photoperiodic control of leaf-growth under natural conditions in Southern England, but this effect is likely to be important in relation to provenance problems.

3. The effect of day-length upon internode-extension appears to be a direct one, and is not effected indirectly through the leaves, as is the case for photoperiodic induction of flowering in herbaceous species.

4. The significance of these results is discussed.

References

- Bünning, E.: Lehrbuch der Pflanzenphysiologie 2. Die Physiologie des Wachstums und der Bewegung. — Berlin, 1939.
- Cailachjan, M. C.: Compt. Rend. Acad. Sci. U.R.S.S. 10: 89. 1936.
- Dostal, R.: (Photoperiodic experiments in *Circaea intermedia* and *Bryophyllum crenatum*). — Vestnik Kral. Ceske Spolecnosti Nauk. Tr. Mat. Prirod. 31: 1. 1944 (Biol. Abst. 12581/49).
- Garner, W. W.: »Photoperiodism». — Biological Effects of Radiation, McGraw Hill Book Co. N.Y. 1936.
- Gregory, F. G.: The Control of Flowering in Plants. — SEB Symp. 2: 75. 1948.
- Harder, R. & Westphal, M.: Jb. Wiss. Bot. 91. 1944 (Cited by Gregory, 1948).
- von Karschon, R.: Untersuchungen über die physiologische Variabilität von Föhrenkeimlingen autochthoner Populationen. — Mitt. der Schweiz. Anstalt f. d. forstliche Versuchswesen. 26: 205. 1949.
- Langlet, O.: Photoperiodismus bei der gemeinen Kiefer (*Pinus silvestris*). — Medd. från Statens Skogsförsöksanst. Stockh. 33: 295. 1942—3.
- Mann, K. L.: Effect of some environmental factors on floral initiation in *Xanthium*. — Bot. Gaz. 102: 339. 1940.
- Priestley, J. H.: Light and Growth I. The effect of brief light exposure upon etiolated plants. — New Phyt. 24: 271. 1925.
- Tolsky, A. P.: Trans. of Forest Experimental Stations. St. Petersburg. 47. 1913. (Abstract in Forest Quart. 12: 227. 1914.)
- Wareing, P. F.: Photoperiodic Control of Leaf Growth and Cambial Activity in *Pinus silvestris*. — Nature 163: 770. 1949.
- Growth Periodicity in *Pinus silvestris*, with special reference to the effect of day-length. — Ph. D. Thesis, University of London, 1950 a.
- Growth Studies in Woody Species I. Photoperiodism in First Year Seedlings of *Pinus silvestris*. — Physiol. Plant. 3: 258. 1950.
- Wight, W.: Radial Growth of the Xylem and Starch Reserves of *Pinus sylvestris*. — New Phyt. 32: 77. 1933.

Studies on the Growth Factor Requirements of Pea Roots

By

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The nutritional requirements of roots have been investigated for several years. As a result of this work, a considerable body of information is available about the inorganic and organic nutrition of roots of a number of varieties of plants [Reviews by White, 33, 34]. Important work has also been done on biosynthetic capacity of a few species [Bonner, 3, 6; Dawson, 8]. These studies have revealed interesting patterns in physiological and biochemical differentiation occurring in roots in contrast with stems and leaves.

Although isolated roots of a number of species have been grown at undiminished rate through many transfers [Bonner, 4; White, 30], there is still a large group which has not been successfully grown in culture. The obvious reason for this is that nutrient solutions presently available do not supply one or more essential substances required for growth of these varieties. Such difficult to culture roots provide an excellent source of material for studying closely related strains of a species at the level of enzyme differentiation.

Some strains of roots are certainly more efficient in synthetic capacity than others. Pyridoxine is said, for example, to be highly essential in the nutrition of tomato roots [Robbins, 20]. Apparently none of the roots of tomato varieties tested could synthesize this vitamin. A different situation is found with respect to niacin synthesis. Roots of some strains of tomato apparently have greater ability to fulfill their niacin requirements than others because the several clones from single varieties used responded differently to niacin additions to the nutrient [Robbins, 19; Bonner, 5]. As a final example it may be pointed out that though many roots are incapable of synthesizing vitamin B₁ and are either dependent upon the leaves or

stems for this vitamin, or certain components of it, flax is known to be able to synthesize part of its B₁ requirements [Bonner, 4] and at least one strain of maize has been shown to synthesize this vitamin in its roots [McClary, 16]. The prospect is roots of some species will be found which can produce all their vitamin requirements. Those which are deficient in ability to produce essential parts of enzymes or amino acids will, however, be most useful in determining the pathway of synthesis of certain critical substances.

In preliminary studies with isolated pea roots we obtained results inconsistent with those previously published for this species. Of three varieties grown, one grew well, another fairly well, and the third hardly at all in the nutrient developed by Bonner for pea (var. Perfection). This has led us to a search for possible causes for these results.

Materials and Methods

Method of obtaining roots: Of the three varieties of pea used — World's Record, Telephone, and Perfection — the one employed in the largest number of experiments has been Perfection. The first two varieties were obtained from a local seed supply house and the last was supplied by the St. Anthony, Idaho division of Associated Seed Growers, Inc.

Both mass collections of roots and clonal stock have been used. When large numbers of uniform roots of a single variety were desired, they were readily obtained by the following procedure. The proper amount of seed was placed in an Erlenmeyer flask and covered with a sterilizing solution. This solution consisted of seven per cent sodium hypochlorite plus two drops of Aerosol OT (25 %) per 100 ml. of solution. Following a twenty-minute sterilization period during which the flasks were agitated vigorously on a shaking machine, the seed was washed eight times with sterile water, then allowed to stand in sterile distilled water for one hour; and after this the liquid was drained off in order to get rid of any remaining traces of sodium hypochlorite. Fresh sterile distilled water was then added and the seed allowed to soak for six or eight hours. At this time the seed was plated out on moistened filter paper in sterile Petri plates. Roots were usually of sufficient length for use within 48 hours. One centimeter segments were cut with sharp surgical scissors, and transferred to the proper nutrient.

Only one clone has been employed in the experiments reported here. This was obtained after the manner described by White (1943); and has been maintained in a dark room kept at approximately 22° C. since September 1949. Frequent, usually weekly, transfer has kept this in excellent growing condition.

Method of growing roots: The basic nutrient solutions used have been those developed by White and Bonner. White's solution consisted of: 360 mgs. MgSO₄, 200 mgs. Ca(NO₃)₂, 200 mgs. Na₂SO₄, 80 mgs. KNO₃, 65 mgs. KCl, 16.5 mgs. NaH₂PO₄ · H₂O, 2.5 mgs. Fe₂(SO₄)₂, 4.5 mgs. MnSO₄, 1.5 mgs.

ZnSO₄, 1.5 mgs. H₃BO₃, 0.75 mg. KI, 20 gms. sucrose, 3.0 mgs. glycine, 1.5 mgs. nicotinic acid, 0.1 mgs. pyridoxine, and 0.1 mg. thiamin per liter. Bonner's solution consisted of 1.5 mgs. ferric tartrate, 20 mgs. KH₂PO₄, 65 mgs. KCl, 81 mgs. KNO₃, 36 mgs. MgSO₄ · 7H₂O, 236 mgs. Ca(NO₃)₂ · 4H₂O, 40 gms. sucrose, 0.1 mg. thiamin, 0.5 mg. nicotinic acid, and 0.1 mg. pyridoxine per liter. Pyrex redistilled water was always used to minimize chemical impurities; and pH was always adjusted to 5.4 ± 0.1 before autoclaving. The basic solutions have been modified in the experiments reported here by varying the kind and amount of sugar, by using an additional inorganic nitrogen source in one experiment and by adding other growth factors such as indoleacetic acid and those contained in casein hydrolysate. («Vitamin-Free» Casein Hydrolysate. General Biochemicals, Inc., Chagrin Falls, Ohio, U.S.A.) In addition, microelements (Hoagland's A-Z without TiO₂) were used as a supplement to Bonner's solution in some of the experiments.

Erlenmeyer flasks (125 ml.) proved to be the most satisfactory containers for growing isolated roots. These were filled with 50 ml. of nutrient, plugged with gauze covered cotton plugs and further protected from dust before autoclaving at 15 lbs. pressure for 15 min. with aluminum foil, and were then ready for the 1 cm. long root tips. All of the operations were carried out under aseptic conditions and contamination losses were low. The cultures were allowed to grow in darkness in a room where the temperature was maintained approximately at 22° C.

In most experiments increase in length in mm. per week as well as increase in dry weight have been used as the measure of growth. Either method is apparently satisfactory though the results are not always strictly parallel, as will be shown. In spite of the precautions taken to insure uniform roots in each experiment there was nevertheless variation between individual roots subjected to any one treatment — whether the roots were from a mass collection or from a clone arising from one original root. The reason for this variation is not known. Possibly it was caused by slight alterations in functional activity of adaptive enzymes.

Experimentation

I. Comparison of growth of Perfection pea in Bonner's and White's media

In order to determine which nutrient — Bonner's or White's — was best for growth of the genetic strain of Perfection pea we were using comparative growth rates were determined over an eleven week period. The roots were excised and transferred weekly. During this period it quickly became apparent Bonner's medium was better balanced for this strain of pea than White's

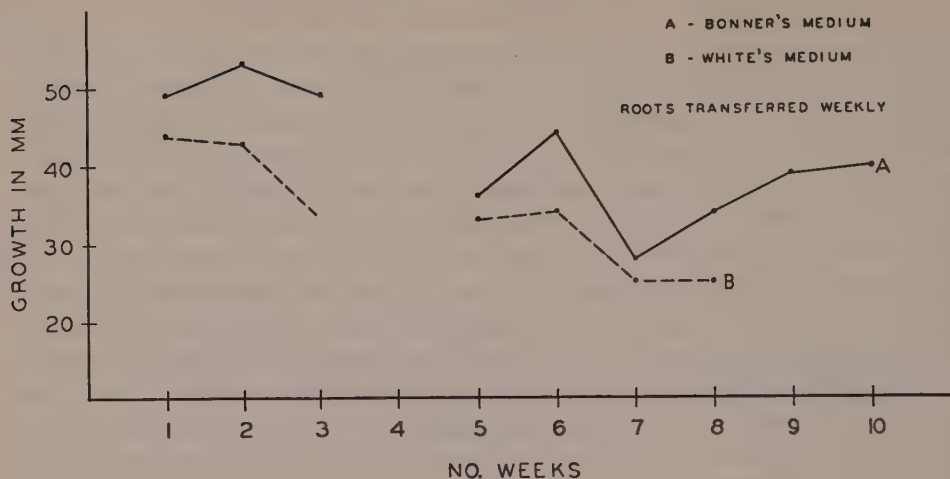


Fig. 1. Growth in length of *Perfection* pea roots in White's and Bonner's media.

solution. There were slight differences by the end of the first week and at no time after that was growth as good in White's medium as Bonner's (fig. 1). At the end of eight weeks the roots grown in White's medium were averaging only 25 mm. per week whereas they were averaging 40 mm. per week in Bonner's medium after eleven weeks.

In another experiment, White's and Bonner's media were used to determine relative growth of unexcised and untransferred roots during a four week period. The results are shown in figure 2. At the end of the first week the roots in Bonner's solution had made the most growth. Apparently at this point some factor or factors became limiting, for the weekly increment thereafter became less and less. A similar decline in growth rate began in the roots grown in White's medium after the second week. This finding for continuous growth in Bonner's medium was later confirmed.

II. Variations in the sugar concentration as a factor in growth of excised pea roots

One of the possible causes of fall-off in growth in Bonner's medium after one week was partial exhaustion of the carbon source. An effort was, therefore, made to determine if higher concentrations than ordinarily used would maintain growth at a high level. Likewise, since it was thought sugar content of Bonner's solution might be the factor limiting growth of excised World's Record and Telephone pea roots in that medium, roots of these

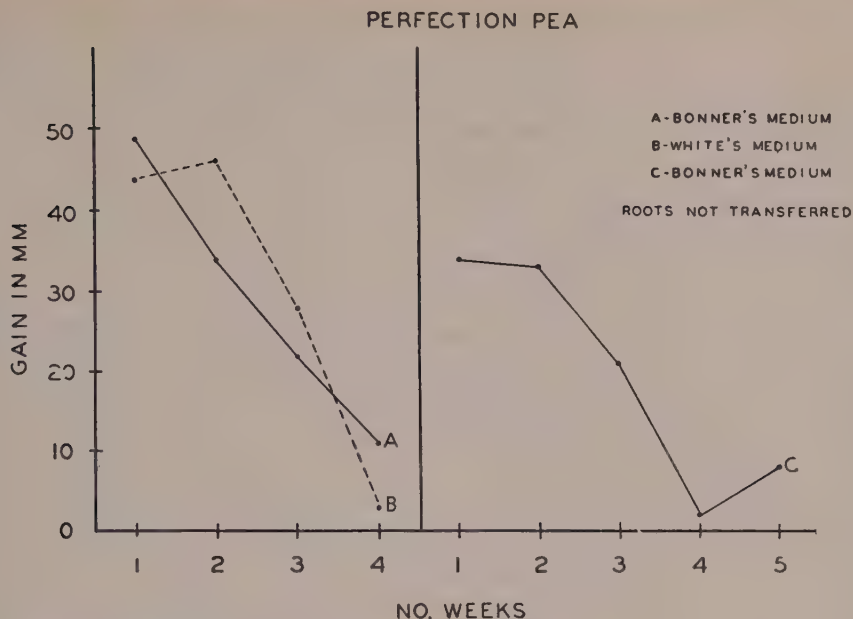


Fig. 2. Rate of exhaustion of a critical factor or factors by Perfection pea roots in Bonner's and White's media.

varieties were grown simultaneously with those of Perfection pea for comparative purposes. The sucrose concentration of the medium was 4 % in one series, 6 % in another and 8 % in the other. Roots were excised and transferred at two-week intervals. The results for Telephone and Perfection over a ten week period are indicated in fig. 3. Growth of Telephone pea roots through the course of the experiment was best when they were supplied with 4 % sucrose, next best with 6 % and least with 8 %. Roots of World's Record peas did not give such clean-cut results as those of Telephone. From the start, there was a steady decline in growth of roots of this variety in all three concentrations of sucrose. Perfection pea roots, in contrast, grew rapidly and produced abundant branch roots with either 6 % or 8 % sucrose in the medium; but with 4 % sucrose, decline in vigor over the ten-week period was continuous.

An additional experiment was performed to determine if dextrose or levulose or a combination of these sugars could act as a suitable carbon source in the growth of Telephone, World's Record and Perfection peas. Eight percent dextrose, 8 % levulose and the combination 2 % dextrose and 2 % levulose were tested. During the first two weeks, roots of World's

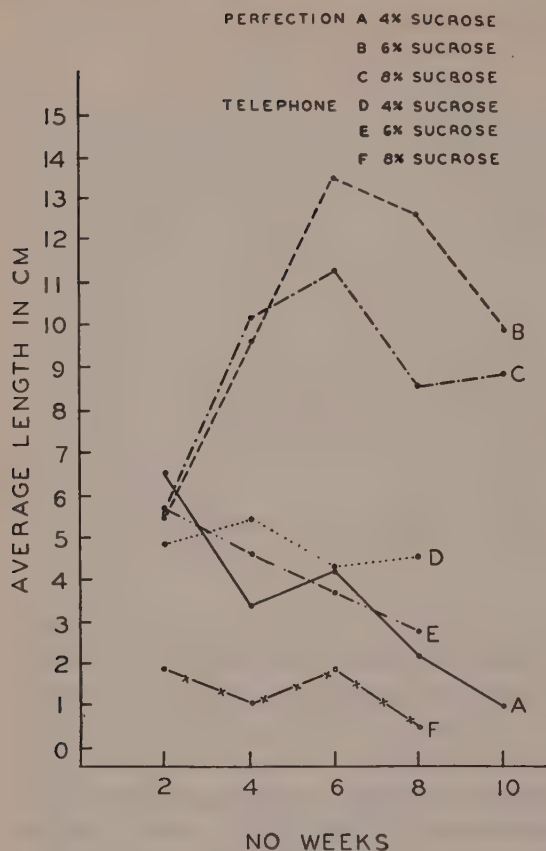


Fig. 3. Effect of addition of sucrose to the basal medium upon average growth of roots of two varieties of peas. The roots were excised and subcultured every two weeks.

Record peas made fair growth on the dextrose-levulose mixture but otherwise none of the solutions supported anything but the most meager growth. After the first transfer, roots of the three varieties grew equally poorly in all of the solutions until the experiment was terminated after running six weeks.

III. Response of excised pea roots to casein hydrolysate with cysteine and tryptophane added to the basal medium

Several experiments have been performed to determine if organic sources of nitrogen can be readily assimilated by pea roots. A »vitamin-free« preparation of casein hydrolysate with L-cysteine and L-tryptophane added was used in concentrations of 200, 400, 800, and 1600 p.p.m. as a supplement to Bonner's solution. The roots were excised and transferred at two-week

intervals over a period of fourteen weeks. Both length and weight were taken as a measure of growth.

Figure 4 shows clearly that casein hydrolysate at concentrations of 800 p.p.m. and above supported as good but no better growth than the controls when measured either on the basis of length increase or weight increase per day. In contrast, 200 and 400 p.p.m. casein hydrolysate supported growth at a much higher level and without the marked fluctuation in growth which occurred in the controls during the $3\frac{1}{2}$ month period. Evidently some component or components of the casein hydrolysate were of value in supporting continuous growth of Perfection pea roots.

Since excellent sustained growth could be obtained by enriching the growing medium with casein hydrolysate and transferring at two week intervals, it was thought some component of this mixture might be the limiting factor noted earlier when attempts to grow roots without transfer were made. Accordingly, the experiment just described was repeated except that transfers were not made but the roots were permitted to grow in the same solution for 5 weeks. Under these conditions greatest growth occurred when there was 100 p.p.m. casein hydrolysate in the medium. Almost as much growth was made when 800 p.p.m. was present; furthermore, the higher concentrations seemed to promote more branching since there was approximately 2.5 times as many branches in this series as any other. Casein hydrolysate at a concentration of 1600 p.p.m. proved to be quite inhibitory, the roots averaging only 2.3 cm. increase in length.

In another casein hydrolysate with L-tryptophane and L-cysteine experiment, Telephone, World's Record and Perfection pea roots were grown in a concentration series ranging from 50 to 1000 p.p.m. Telephone pea grew best with 50 p.p.m. of the supplementary substances in the medium but increase in length was less and less with each transfer in all of the solutions. World's Record pea roots grew satisfactorily for the first time. Concentrations of 100, 200, and 400 p.p.m. all proved to be effective in sustaining growth at a fairly uniform rate. Those roots receiving 100 p.p.m. averaged approximately 7.0 mm. per day. Fifty p.p.m. was apparently insufficient and concentrations of 800 and 1000 p.p.m. were of little value. As in a previous experiment, Perfection pea roots were found to grow most uniformly and most rapidly at concentrations of 200 and 400 p.p.m. Roots in these solutions gained, on an average, a little more than 7 mm. per day.

A clone of a Perfection pea root was established for the purpose of making further studies on growth factor requirements. This root was selected at random from a large population growing in Bonner's solution supplemented with casein hydrolysate. In the course of developing the clone it was found the root tips from the side branches would not grow as rapidly as tips taken

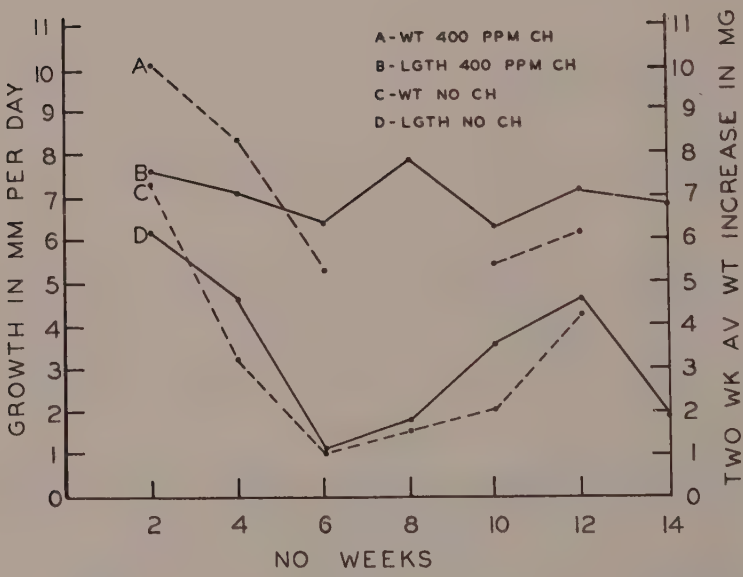
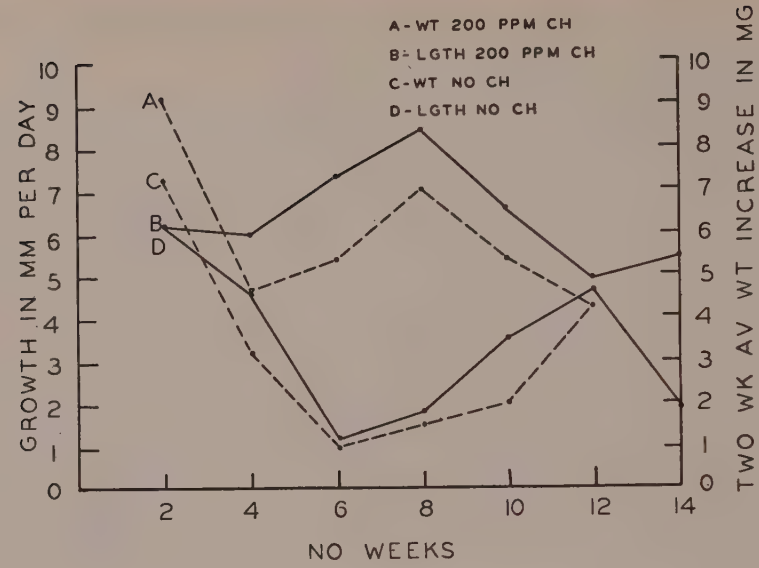


Fig. 4.

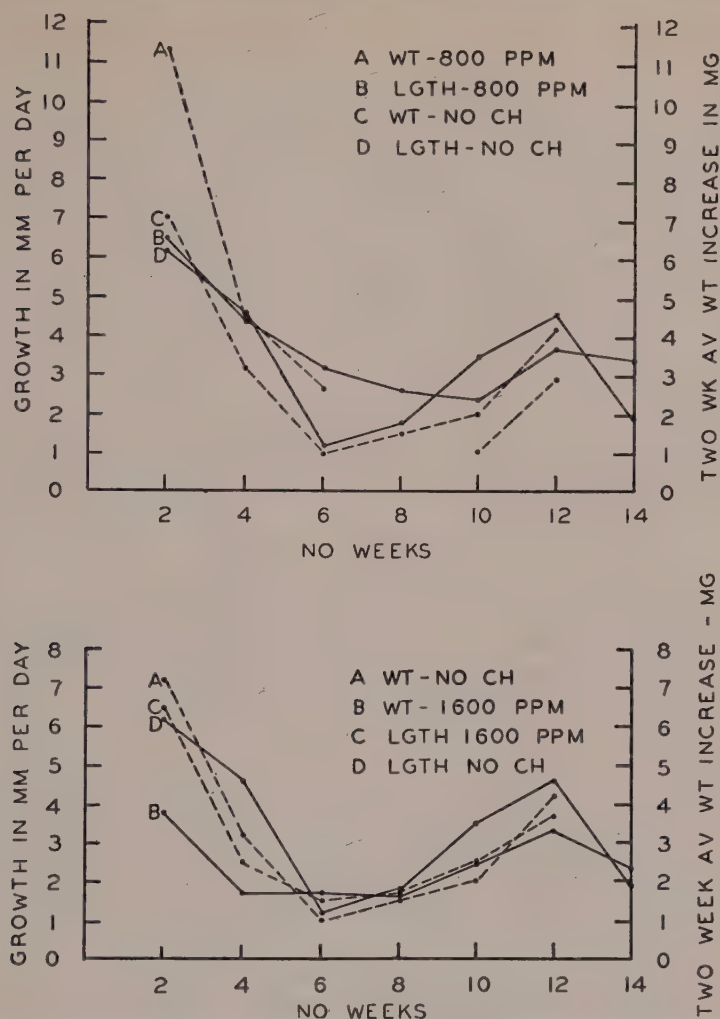


Fig. 4. Growth in length and weight of *Perfection* pea in a nutrient series to which casein hydrolysate and L-cysteine and L-tryptophane were added. Measurements were made at two week intervals. The roots were then excised and 1 cm. tips transferred to fresh solution.

from the primary root; and not until after the fourth weekly transfer were they equivalent. Therefore, only roots whose tips had gone through four transfers and were known to be growing uniformly were used.

The first experiment with this clone was to determine relative rate of growth in Bonner's medium and the same solution supplemented with 400 p.p.m. casein hydrolysate with L-cysteine and L-tryptophane (each at 6.67

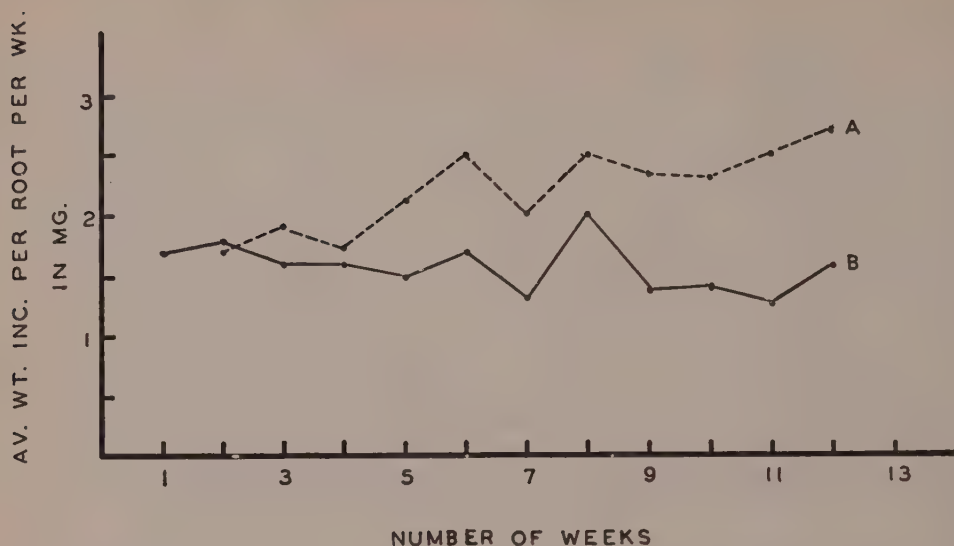


Fig. 5. Average growth of a clone of *Perfection* pea roots on two media. A. Grown on Bonner's medium supplemented with: 1) Hoagland's A—Z solution; 2) casein hydrolysate, 400 p.p.m.; 3) L-cysteine and L-tryptophane (each at 6.67 mg. per 100 mg. casein). B. Grown on Bonner's medium supplemented with Hoagland's A—Z solution.

mg. per 100 mg. casein). This experiment was conducted over a twelve week period and yielded some well defined results. The casein hydrolysate supplemented solution promoted the greatest amount and most uniform growth. Those roots growing in Bonner's medium underwent rigorous natural selection. This was all the more apparent because just one root tip was lost through contamination during the entire experiment. Only one of the 26 roots growing in the supplemented solution stopped growing, while 11 of 27 in the unsupplemented Bonner's solution ceased growing during the three month period. At the conclusion of the experiment 22 of the 24 surviving roots in the supplemented Bonner's solution were growing more than 50 mm. per week, but only nine of the 16 remaining roots in Bonner's solution were growing more than this much. Five of these, however, were apparently well adapted and were growing exceptionally well. The general superiority of the supplemented Bonner's medium over the unsupplemented is indicated in fig. 5 where average weight values are shown. The gradual decline in average weight increase per week by the roots growing in Bonner's solution is probably in part a reflection of slow exhaustion of some factor stored while growing in the supplemented solution.

IV. *Response of excised pea roots to supplementary inorganic nitrogen, L-cysteine and L-tryptophane*

Inasmuch as the nitrogen content of the basal solution had been increased by the use of the casein hydrolysate — L-cysteine — L-tryptophane supplement, it was thought the increase in growth obtained with these additions may have been induced by this fraction. A determination of the effect of nitrogen (roughly equivalent to the amino nitrogen in 300 p.p.m. of casein hydrolysate) was made with the addition of NH_4Cl at 108 p.p.m. to the medium. Roots of Telephone, World's Record, and Perfection pea roots were all tried. These were excised and transferred every other week. The fact NH_4Cl was not a suitable supplementary nitrogen source and was indeed toxic became apparent immediately. All of the roots in the test solutions grew poorly — below 4 mm. per day. Because of this, the experiment was terminated after six weeks.

Two amino acids were tested — L-cysteine and L-tryptophane — in combination as additions to Bonner's medium. Five concentrations of the combination were used, the highest being equivalent to that employed in previous experiments with casein hydrolysate, the lowest being one-twentieth of this amount. Roots of all three varieties were subjected to test. Telephone pea roots grew very poorly while roots of Perfection and World's Record made good growth in some concentrations. Although growth of Perfection pea roots was not as great in any of the five concentrations as in the best nutrients developed, it was 60 % greater in two concentrations of the L-cysteine and L-tryptophane mixture than in Bonner's solution alone. Moreover, roots of World's Record pea made sustained growth in the supplemented mixture, whereas they grew quite slowly in Bonner's unsupplemented solution.

V. *Response of excised pea roots to indoleacetic acid*

Indoleacetic acid has been found by Gautheret and his co-workers to be essential for maximum growth of tumorous tissues; but since root requirements for this substance are low and may be met by local synthesis, indoleacetic acid is not ordinarily used in the medium where they are grown. An experiment was performed, however, to determine the effect of this substance on growth rate of our Perfection pea root clone. All of the roots used in this experiment had been subcultured a number of times and were growing quite uniformly when subjected to treatment. Bonner's medium and Bonner's medium plus 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} and 10^{-8} gm./liter of indoleacetic acid were used. During the six weeks this experiment was run the roots were measured, excised and transferred weekly. Addition of indole-

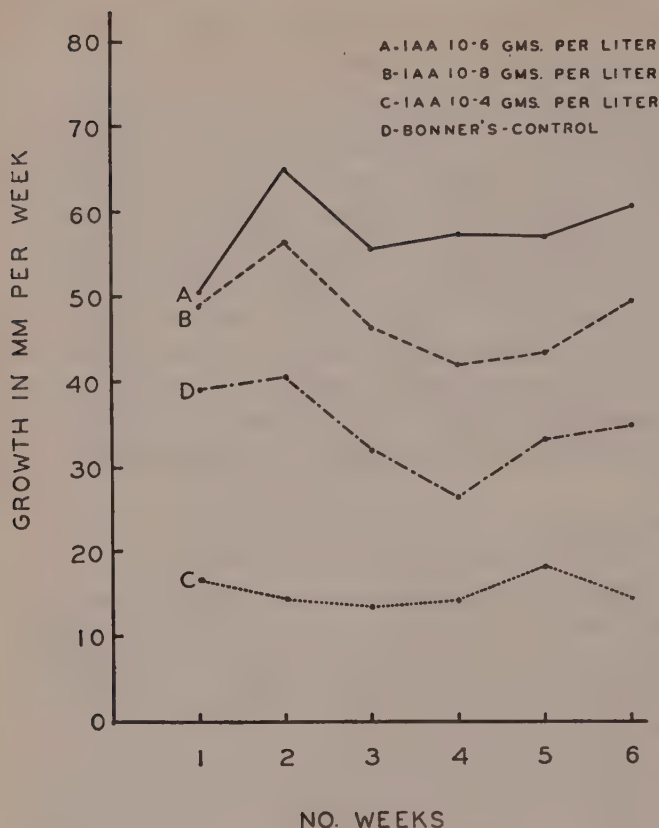


Fig. 6. Effect of addition of indoleacetic acid (IAA) to the basal medium upon growth of isolated *Perfection* pea roots.

acetic acid to the basal medium at the rate of 10^{-4} gm./l. resulted in symptoms of toxicity — the roots being irregularly thickened along their length, and having numerous closely spaced secondaries all of which were somewhat swollen at the tip. Roots growing in the solution supplemented with indoleacetic acid at 10^{-5} gm./l. grew on an average 3.0–3.5 cm. per week and were thick in diameter with very little branching. The best growth achieved was with 10^{-6} gm./l. indoleacetic acid — roots in this solution growing an average of 5.5 cm. per week (fig. 6). Appearance of the roots was excellent. The main root was of good diameter and well branched. Overall growth in this solution was quite comparable to that in Bonner's solution with 400 p.p.m. casein hydrolysate plus L-cysteine and L-tryptophane. Both 10^{-7} and 10^{-8} gm./l. indoleacetic acid were ineffective in promoting growth. Those roots receiving 10^{-7} gm./l. grew an average of 3.7 to 4.0 cm. per week and although there was some branching, it was less than when 10^{-6} gm./l. indoleacetic acid was present. With 10^{-8} gm./l. indoleacetic acid

in the medium the roots gained an average of 3.0 to 3.5 cm. per week, were thin and branched very little. These findings were confirmed in a repetition of this experiment.

VI. *Effects of coconut milk and banana diffusate upon growth of isolated Perfection pea roots*

Because of the possible presence of growth factors in coconut milk and banana fruit which might be of value in promoting growth of isolated roots, experiments were designed to test both sources. In testing coconut milk, Bonner's medium was made up as usual except different portions of the liquid were derived from mature coconuts. Three concentrations — 5 %, 15 %, and 30 % — were derived in this way. Before autoclaving the pH was adjusted as usual to 5.4. No growth occurred in any of the roots. This may have been caused by a naturally occurring inhibitor, a toxic product formed during autoclaving, or by too high a concentration of a growth promoting substance — which, has not been determined.

Banana diffusate was tested similarly. It was prepared by soaking banana powder in 10 times its weight of water, blending for a few minutes in a Waring Blendor, centrifuging and decanting the supernatant. Bonner's medium was then made up to consist of 1.9, 2.0, and 4.0 % banana extract. Toxic action was noted within the first 24 hours. By the end of the first week a large number of roots were dead and those which were alive had not grown appreciably. It must, therefore, be concluded that if growth factors are present in banana diffusate they either cannot be used by Perfection pea roots or were present in inhibitory quantities at the concentrations used.

Discussion

The metabolism of roots of closely related varieties of peas differs greatly. Synthesis of necessary growth factors either does not occur in certain of these or they are produced so slowly that they quickly become limiting factors in growth. High growth rates of Perfection pea may be obtained in several ways as is shown in table 1. A growth rate of slightly more than 7 mm. per day was obtained by using either 6 % sucrose or 200—400 p.p.m. casein hydrolysate or 10^{-6} gm./l. indoleacetic acid as supplements to the basal medium. Although continuous growth of Perfection pea roots can be obtained on Bonner's medium alone, this is accomplished by rigorous selection which results in the survival of only a few roots from a large initial population. These survivors may grow quite well. Much more uniform growth, however, can be obtained in a mass population of roots if casein

Table 1. *Growth of isolated Perfection pea roots in Bonner's solution when sugar concentration is varied or it is supplemented with casein hydrolysate plus L-cysteine and L-tryptophane or with indoleacetic acid.*

Medium		Av. Growth Rate mm. per day	No. Weeks	Origin of Roots	Period of Transfer Weeks
Sucrose	4 0/0	5.02	12	clonal	1
	4 0/0	2.93	24	non-clonal	2
	6 0/0	7.44	10	» »	2
	8 0/0	6.59	10	» »	2
Casein Hydrolysate	50 p.p.m.	6.12	12	» »	2
	100 p.p.m.	5.99	18	» »	2
	200 p.p.m.	7.18	26	» »	2
	400 p.p.m.	7.20	26	» »	2
	400 p.p.m.	7.25	12	clonal	1
	800 p.p.m.	3.25	20	non-clonal	2
	1000 p.p.m.	4.12	6	» »	2
	1600 p.p.m.	2.30	14	» »	2
Indoleacetic acid	10 ⁻⁴ gm./l.	0	4	clonal	1
	10 ⁻⁵ gm./l.	4.58	5	»	1
	10 ⁻⁶ gm./l.	7.70	5	»	1
	10 ⁻⁷ gm./l.	5.64	5	»	1
	10 ⁻⁸ gm./l.	4.74	5	»	1

hydrolysate with L-cysteine and L-tryptophane are added to the medium, and certainly a higher percentage of roots survive and grow well if the basal medium is so supplemented. In contrast with this, World's Record pea roots showed no tendency to grow at all in the unsupplemented medium, though it is probable if a sufficiently large population were grown a few roots could be selected which would grow in the basal medium. No evidence is available at present, however, which indicates such selections could be made from a population of Telephone pea roots. Evidently some critical enzyme or enzymes involved in the synthesis of essential metabolites function at a very low level in these roots. Because of this these roots offer excellent material for study of natural genetic blocks in synthesis.

The kind and amount of sugar readily used as a carbon source varies from species to species. Dextrose supplied to isolated sugar cane roots is quickly converted to sucrose [Hartt, 15], but not all roots appear able to use dextrose. White (32) and others have shown that sucrose is more readily used by tomato roots than is dextrose; however, dextrose, levulose, maltose and cellobiose are utilizable as carbon sources [Robbins and Schmidt, 21; Dormer and Street, 10]. Sucrose is also the best source of carbohydrate for the growth of excised cotton roots, dextrose is next best, and levulose is inhibitory [Wilson, 29]. Maize roots, on the other hand, utilize maltose more

readily than other sugars and show most inhibition on galactose and lactose [Würgler, 35]. Possibly the superior growth obtained with maltose may be attributed to impurities of a non-carbohydrate nature. Our strains of Perfection and Telephone pea roots were unable to utilize either dextrose or levulose sufficiently rapidly to support growth. World's Record roots, on the other hand, did have some ability to use a mixture of dextrose and levulose. But capacity for utilization eventually became a limiting factor in their growth. Sucrose, however, appeared to be utilized by roots of all three varieties.

Sucrose requirements vary from species to species and from variety to variety. White found 2 % sucrose most suitable for his clones of tomato roots, but Glasstone (14) found 1 % just as satisfactory as higher concentrations for hers. Roots of different varieties of peas are equally variable in their sugar requirements, those of Perfection pea growing most rapidly on 6 and 8 % sucrose while those of Telephone pea grow best with 4 % in the medium and seemed to be inhibited by higher concentrations. Only a comparatively small amount of sugar is used by tomato roots during the interval between transfers and it is difficult to understand why concentrations above 1 or 2 % apparently become limiting in the growth of pea roots.

Ammonium nitrogen has been shown to be utilized rapidly by some plants under optimal conditions of growth. Robbins and Schmidt (21) found excised tomato roots could not utilize either NH_4Cl or $(\text{NH}_4)_2\text{SO}_4$ unless some vitamin B_1 was present; even then growth was not as rapid as when NO_3 was the nitrogen source. Nitrate also seems to be the most satisfactory source of inorganic nitrogen for pea roots, this source being superior to nitrate plus ammonium chloride in supporting growth of Perfection, World's Record and Telephone pea roots.

A few experiments have indicated that amino acids can be utilized by higher green plants as a source of nitrogen. In most cases where they are utilized they are found of value as supplements to the inorganic nitrogenous salts. Immature *Datura* embryos, for example, apparently can utilize amino acids faster than they can synthesize them [Sanders and Burkholder, 22]. In attempts to determine which factors are supplied by leaves in the rooting of cuttings Doak (9) demonstrated that a mixture of amino acids promoted root formation in *Rhododendron* and later van Overbeek *et al* (27) found that arginine and serine were both effective in promoting rooting of leafless *Hibiscus* cuttings. Sharp differentiation between root initiation and development was not made by these workers.

Some investigations have been made upon the effect of individual and combinations of amino acids upon the growth of isolated tomato [Robbins and Schmidt, 21; White, 31; Bonner, 5] and pea roots [Addicott and Bon-

ner, 1]. White's finding that glycine is of benefit in the culture of tomato roots has not been substantiated by either Robbins or Bonner. Since legumes are better able to use organic nitrogen than other groups [Virtanen and Linkola, 28] they present favorable material for studying relative rate of synthesis and use of these substances. Addicott and Bonner, in their studies with pea roots concluded »Although the data presented . . . do not yet permit of a definitive decision, still it seems possible that the amino acid mixture is not essential to the continued optimal growth of isolated pea roots.« In our studies with Perfection pea roots, however, twice as much growth was obtained when Bonner's medium was supplemented with 200—400 p.p.m. casein hydrolysate as with the unenriched medium. Although a »vitamin-free« preparation of casein hydrolysate was used, it is not known whether traces of substances other than amino acids were the effective constituents. Extensive trials are in progress to test this point. A combination of cysteine and tryptophane has already given indications of exerting some promotive effect.

Whether or not indoleacetic acid inhibits or stimulates growth of isolated roots has been a much disputed point. Analyses of distribution of auxin in naturally growing *Avena* roots have shown there is relatively little present [Thimann, 24], most being in the tip with lesser amounts found proximally. Some doubt has been entertained about the ability of roots to synthesize this substance; but Nagao (17) demonstrated with *Helianthus annuus* root tips that even after a number of transfers auxin was in the tips and suggested synthesis was taking place there. Later, he correlated auxin production in the pea root with growth [Nagao, 18]. Quantitative measurements of auxin production by isolated Perfection pea roots have shown [van Overbeek, 25, 26] that though the auxin content of pea root tips decreased noticeably within two weeks after excision, they continued to produce some auxin throughout the 34 week period of the experiment.

Certain it is, relatively small amounts of indoleacetic acid and other growth substances can cause inhibition of root growth. Possibly roots of some varieties synthesize their necessary auxin as rapidly as they can use it. No stimulation of isolated tomato roots by indoleacetic acid was observed by Robbins and Schmidt (21), by Friedman and Francis (12), or by White (33, p. 188) and only slight stimulation was noted by Geiger-Huber and Burlet (13). Stimulation in this case was within the range of probable error of the experiment and hence of doubtful significance. Isolated maize roots have been reported, a number of times, to be stimulated by indoleacetic acid. Fiedler (11) obtained slight stimulation; Geiger-Huber and Burlet found that 10^{-10} to 10^{-13} molar indoleacetic acid was stimulatory with a maximum at 3×10^{-11} molar; the variety Tauja-Thielman and Pelece (23) were work-

ing with showed some stimulation at 1:200,000,000; Würdler (35) reported stimulation of growth in maize with several growth substances other than indoleacetic acid; and, more recently, Bein and Schopfer (2) have reported stimulation in growth of isolated maize roots with 10^{-11} molar 2,4-dichlorophenoxyacetic acid. Effects of indoleacetic acid upon isolated pea roots have also been studied. Bonner and Koepfli (7) found a slight stimulation of root growth with auxin concentrations between 10^{-8} and 10^{-10} molar in contrast with complete inhibition at all concentrations when *Avena* roots were used as the test object. Since our clone of Perfection pea root is stimulated to a greater than 50 % increase in growth rate over the controls by 10^{-6} grams/liter indoleacetic acid in the medium, it would appear that this root can utilize more indoleacetic acid than is produced in the tips when grown in Bonner's medium without this substance.

Summary

1. Attempts have been made to grow isolated roots of three varieties of pea — World's Record, Telephone, and Perfection — in culture for six or more weeks.

2. Neither Bonner's nor White's medium supported maximum growth of these varieties. Perfection pea roots made the most growth, World's Record was next, and Telephone pea grew least of all. With mass populations of roots of all three varieties, however, decline in growth was continuous with time.

3. The amount of sucrose in the nutrient markedly influenced growth of the roots. Perfection pea roots grew most rapidly with 6 and 8 % sucrose in the medium; Telephone pea roots grew most vigorously with 4 %; but results with World's Record roots were not decisive.

4. Dextrose and levulose separately and in combination did not prove to be satisfactory sources of carbon for growth of isolated roots of any one of the three varieties tested.

5. Casein hydrolysate together with L-cysteine and L-tryptophane (each at 6.67 mg. per 100 mg. casein) was found to be an excellent supplement to Bonner's solution when added at the rate of 200 and 400 p.p.m. Perfection pea roots grew very well at these concentrations but at concentrations above this, 800 and 1600 p.p.m., growth was no greater than the controls. World's Record pea roots were grown satisfactorily for the first time, concentrations of 100, 200, and 400 p.p.m. proving most effective in sustaining growth at a uniform level. Roots of Telephone pea still could not be maintained satisfactorily.

6. L-Cysteine and L-tryptophane in combination supported growth of Per-

fection and World's Record pea roots at a level above the controls, but growth of roots of Telephone pea was not sustained.

7. With casein hydrolysate added to the basal medium some factor or factors became limiting in the growth of Perfection pea roots within 12 days. This made it necessary to transfer the roots to fresh medium each week.

8. Indoleacetic acid added to the basal medium was very effective, in a narrow range, in promoting uniform and high growth rates of Perfection pea roots. Although indoleacetic acid at 10^{-4} gm./l. was toxic, concentrations of 10^{-7} and 10^{-8} were insufficient to stimulate growth significantly. Greatest promotive activity was induced by indoleacetic acid at 10^{-6} gm./ liter.

9. Coconut milk and banana fruit diffusate both proved ineffective in stimulating growth of Perfection pea roots. Both caused marked inhibition at all concentrations employed.

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References

1. Addicott, F. T., & Bonner, J.: Nicotinic acid and the growth of isolated pea roots. — *Science* 88: 577. 1938.
2. Bein, M. & Schopfer, W. H.: Sur l'action de l'acide 2,4-dichlorophénoxyacétique sur la croissance des racines de *Zea mays* et de *Pisum* cultivées *in vitro*. — *Experientia* 4: 222. 1948.
3. Bonner, J.: Thiamin (Vitamin B₁) and the growth of roots: the relation of chemical structure to physiological activity. — *Amer. Jour. Bot.* 25: 543. 1938.
4. — On the growth factor requirements of isolated roots. — *Amer. Jour. Bot.* 27: 692. 1940.
5. — Further experiments on the nutrition of isolated tomato roots. — *Bull. Torrey Bot. Club* 70: 184. 1943.
6. Bonner, J., & Buchman, E. R.: Syntheses carried out *in vivo* by isolated pea roots. I. — *Proc. Nat. Acad. Sci. (U.S.A.)* 24: 431. 1938.
7. Bonner, J., & Koepfli, J. B.: The inhibition of root growth by auxins. — *Amer. Jour. Bot.* 26: 557. 1939.
8. Dawson, R. F.: Nicotine synthesis in excised tobacco roots. — *Amer. Jour. Bot.* 29: 813. 1942.
9. Doak, B. W.: Amino acids and rooting of cuttings. — *Nature* 144: 379. 1939.
10. Dormer, K. J., & Street, H. E.: The carbohydrate nutrition of tomato roots. — *Ann. Bot. N.S.* 13: 199. 1949.
11. Fiedler, H.: Entwicklungs- und reizphysiologische Untersuchungen an Kulturen isolierter Wurzelspitzen. — *Zeit. Botan.* 30: 385. 1936.
12. Friedman, B. A., & Francis, T., Jr.: Gall formation by *Phytophthora tumefaciens* extract and indole-3-acetic acid in cultures of tomato roots. — *Phytopath.* 32: 762. 1942.
13. Geiger-Huber, M., & Burlet, E.: Über den hormonalen Einfluss der Beta-indolyllessigsäure auf das Wachstum isolierter Wurzeln in Keimfreier Organkultur. — *Jahrb. Wiss. Bot.* 84: 233. 1936.

14. Glasstone, Violette F. C.: Inorganic micronutrients in tomato root tissue culture. — Amer. Jour. Bot. 34: 218. 1947.
15. Hartt, Constance E.: (Report of) Enzyme Laboratory. — Proc. Hawaiian Sugar Planters' Assoc., Rept. Comm. in Charge Exp. Sta. 59: 113. 1939.
16. McClary, J. E.: Synthesis of thiamin by excised roots of maize. — Proc. Nat. Acad. Sci. (U.S.A.) 26: 581. 1940.
17. Nagao, Masayuki: Growth hormones of plants. III. Occurrence of growth substances in isolated roots grown under sterilized conditions (Preliminary report). — Sci. Repts. Tohoku Imp. Univ., Fourth Ser. 12: 191. 1937.
18. — Growth hormones of plants. IV. Further experiments on the production of growth substance in root tips. — Science Repts. Tohoku Imp. Univ., Fourth Ser. 13: 221. 1938.
19. Robbins, W. J.: Growth of excised roots and heterosis in tomatoes. — Amer. Jour. Bot. 28: 216. 1941.
20. — Specificity of pyridoxine for excised tomato roots. — Amer. Jour. Bot. 29: 241. 1942.
21. — & Schmidt, Mary B.: Growth of excised roots of the tomato. — Bot. Gaz. 99: 671. 1938.
22. Sanders, Mary E., & Burkholder, P. R.: Influence of amino acids on growth of *Datura* embryos in culture. — Proc. Nat. Acad. Sci. (U.S.A.). 34: 516. 1948.
23. Tauja-Thielman, M. J., & Pelece, E.: Wachstum isolierter Wurzelspitzen in Heteroauxin enthaltenden Nährlösungen. — Arch-Exptl. Zellforsch. bes. Gewebezücht. 24: 1. 1940.
24. Thimann, K. V.: Studies on the growth hormone of plants. VI. The distribution of the growth substance in plant tissues. — Jour. Gen. Physiol. 18: 23. 1934.
25. van Overbeek, J.: Evidence for auxin production in isolated roots growing in vitro. — Bot. Gaz. 24: 450. 1939.
26. — Is auxin produced in roots? — Proc. Nat. Acad. Sci. (U.S.A.). 25: 245. 1939.
27. —, Gordon, S. A., & Gregory, L. E.: An analysis of the function of the leaf in the process of root formation in cuttings. — Amer. Jour. Bot. 33: 100. 1946.
28. Virtanen, A., & Linkola, H.: Organic nitrogen compounds as nitrogen nutrition for higher plants. — Nature 158: 515. 1946.
29. Wilson, Coyt.: Some factors affecting the growth of excised cotton roots. — Proc. Assoc. Southern Agr. Workers. 42: 209. 1941.
30. White, P. R.: Cultivation of roots of dicotyledonous plants. — Amer. Jour. Bot. 25: 348. 1938.
31. — Glycine in the nutrition of excised tomato roots. — Plant Physiol. 14: 527. 1939.
32. — Sucrose vs. dextrose as carbohydrate source for excised tomato roots. — Plant Physiol. 15: 355. 1940.
33. — A handbook of plant tissue culture. — Jaques Cattell Press, Lancaster, Pa. 1943. 277 pp.
34. — Plant tissue cultures II. — Bot. Rev. 12: 521. 1946.
35. Würgler, W.: Growth of roots of *Zea mays* in organ culture and the influencing of it by hormones. — Ber. Schweiz. Botan. Ges. 52: 239. 1942.

Some Observations on the Influence of 2,4-Dichlorophenoxyacetic Acid (2,4-D) on the Growth and Development of Two Varieties of Wheat.

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Introduction

Recent investigations indicate that apart from their extensive application as herbicides and in modifying dormancy, abscission, bud differentiation, fruit set etc., synthetic growth substances may also have far-reaching effects on reproduction and growth pattern in plants. Excellent reviews on the subject are available in the literature and only a few investigations more or less pertinent to the observations made by the authors will be briefly referred to.

Modification of the pattern of new organs brought about by application of growth substances has been reported by Zimmerman (1943). Substituted phenoxy, benzoic, and naphthoxy acids were found to be particularly effective for this purpose. Fully mature parts did not change their shape, but new growth occurring after treatment was modified. Aberg and Denward (1947) found several abnormalities in barley spikes when the crop was sprayed with growth substances. These were: extended rachis internodes, increased number of kernels at some of the rachis nodes and widened lemma with more than one awn. They pointed out similarity between these abnormalities and those of genetic origin and suggest that there is a resemblance between the hormone derivatives and the substances that the genes produce to direct the development and structure of plants.

Differential varietal response to, and a carry-over effect on the succeeding crop, of the application of growth regulators has also been observed by several workers. Kaiser and Albaum (1939) found that oat seedlings of an early and late type, respectively, grown in the dark for a period of 4 to 5 days responded differently to 3-indole acetic acid. Rossman and Staniforth (1949) found that some inbred

lines of corn were more susceptible than others to rolling of leaves, inhibition of brace-root development and defective tassel formation when 2,4-D was applied to foliage at the 6- to 8-leaf stage. The air dry weights per 100 kernels were reduced as a result of 2,4-D application to parent plants in all the cases. The total germination of seeds harvested from sprayed plants was not reduced in any line but the percentage of weak seedlings was significantly greater than in seed from unsprayed plants. Rossman and Sprague (1949) also found that in some cases there was a carry-over effect on the succeeding crop after spraying corn plants with 2,4-D, and significant reduction in yield occurred. Clark and Witter (1949) also observed that the hastening of seed stalk elongation, in lettuce and celery, induced by the application of growth substances was transmitted to the progeny of treated plants. Aberg and Denward (1947) did not, however, find any abnormalities inherited in two generations of crops raised successively from the seeds of the original crop.

Leopold (1949) observed that by artificially, augmenting the auxin supply in the leaves tillering was decreased in barley, whereas it was increased by the application of agents known to destroy or oppose the action of auxin in growth (TIBA, X-rays and coumarin). Leopold and Thimann (1948) report that low concentrations of indoleacetic acid which had an inhibitory effect on vegetative buds (tillering) increased the number of flower primordia in wintex barley under long day (which is necessary to induce flowering in this variety).

So far as the writers are aware, a study of the effect of growth substance on growth, development and nutrition of the plant, during its complete life cycle, has not been attempted, at least under Indian conditions. An investigation was, therefore, undertaken with two varieties of wheat viz. NP 165, an early vulgare type and S 40, a late durum. When sown at the normal time in early November at Delhi the ears emerge in NP 165 after about 13 weeks, whereas in S 40 they emerge later by 6 weeks. The grains in S 40 are not wellfilled and plump as they mature during the hot and dry weather in April and its yield is, therefore, much lower.

Experimental procedure

The plants were grown in water (in glass containers, about 15 lit. capacity each) as well as soil culture (in unglazed earthen pots). The choice of a suitable concentration of 2,4-D was made after a preliminary trial on the root growth of two-day old seedlings studied over a period of five days. Concentration of 1 ppm proved too toxic as the plants dried up ultimately with little root growth. In 0.25 ppm concentration the final root length reached was about 30 % of that in water, and this slightly toxic concentration was decided upon for investigation with a view to ensuring that it was well within the effective range. Experiments with several concentrations, although desirable, were not taken up on account of unavoidable circumstances.

Hoagland's culture solution No. 1, at one-third the normal strength, (Hoagland and Arnon 1938), was used in the water culture. Boron, manganese, zinc and copper were also added as recommended by them. Tap water was used for making up the culture solution. The solution was changed once a fortnight and the pH was adjusted to 6.4 from time to time. The proper quantity of 2,4-D was also added to the culture solution whenever it was changed.

The seeds were kept for germination between folds of wet muslin cloth on 24th November, 1948 and uniform seedlings were transplanted to culture vessels a week later. The seedlings were allowed to establish themselves for another 4 days and then a requisite quantity of sodium salt of 2,4-D was added, to bring its concentration to 0.25 ppm, to half the number of culture vessels.

Plant samples were collected from water cultures for the determination of dry weights and nitrogen content according to the following schedule:

Sample Number	Date	Days after sowing	Numbers of plants			
			NP 165		S 40	
			Control	2,4-D	Control	2,4-D
1	5.1.49	42	30	60	30	60
2	4.2.49	72	20	40	20	40
3	29.3.49	126	—	—	5	5
4		Harvest	21	22	18	16

Ten plants taken at random were dissected out on each occasion to examine the growing point. The material from these and the remaining plants was then separated into green leaves, roots and shoot (including sheaths, stem, dry leaves and green leaves not fully unfolded) and dried in an oven. Total nitrogen content of the dry powdered material was determined by the Kjeldahl method after reducing nitrate by salicylic sulphuric acid mixture.

Seedlings were transplanted to pots under soil culture on 2/12/48. Five seedlings were kept in each pot, holding about 22 lb. of air-dry soil. A set of six pots was allotted to control and 2,4-D treatment respectively. Immediately after transplanting the dry soil was watered with two litres of water, the six treatment pots receiving in addition 2 mg. of 2,4-D dissolved in it. Ten days later, each pot was manured with 2 gm. ammonium sulphate, 1 gm. potassium sulphate and 1 gm. superphosphate. Another mg. of 2,4-D was added when the plants were 8 weeks old. Finally only the pots under the variety S 40 were treated again with another mg. of 2,4-D when the plants were about 15 weeks old. As the field capacity of the soil was about 15 % the aqueous concentration of 2,4-D was about 1 ppm to start with but the effective concentration would be much lower as the substance gradually decomposed. Observations on tillering, leaf number and final grain yield only were taken on these plants.

Both the culture vessels and the pots were kept in the open in direct sunlight, the roots in the culture vessels, however, being protected against light by suitable shading.

Observations from dissections on main shoots of plants from water culture

The observations made on 5.1.49, 4.2.49 and 29.3.49, 42, 72 and 126 days, respectively, after sowing are recorded in table 1. Considering the variety NP 165 first, it will be seen that the rate of development of the plants treated with 2,4-D was generally slower. The reproductive phase, as judged by the initiation of double ridges, had already commenced before the first observation was made. The total number of leaves differentiated was 9.0 in the control and 8.6 in the treated plants. Tiller production was, however, much more adversely affected by 2,4-D, the total number differentiated being 6.4 in the control and only 2.8 in the treated plants. The negligible effect of 2,4-D on leaf differentiation can perhaps be accounted for by the fact that nearly half the number of leaves is already differentiated in the embryo.

The elongation of the stem was not influenced by 2,4-D, but the number of double ridges on the growing point was very much reduced.

Observations on the 72nd day after sowing revealed considerable reduction due to treatment in the number of tillers, fully unfolded leaves and spikelets and also in the length of stem and ear. Various deformities in the leaves and spikelets were also noticed in 8 plants out of 10. At this time several deformities were also noticeable in the external morphology of the treated plants, which can be conveniently described by referring to a typical plant (fig. 1). The flag leaf (8th) was irregular in growth and was not fully

Table 1. *Observations from dissection on main shoots. Mean of 10 plants.*

Date of observation	NP 165				S 40					
	5.1.49		4.2.49		5.1.49		4.2.49		29.3.49	
	C ¹	2,4-D	C	2,4-D ²	C	2,4-D	C	2,4-D	C	2,4-D
Tiller Number	3.5	1.0	5.2	1.7	3.5	1.6	5.6	3.3	3.0	3.6
No. of tiller buds	2.9	1.8	—	—	1.8	3.1	1.0	1.9	—	—
Number of fully unfolded leaves	5.4	4.6	8.8	5.9	5.2	4.9	7.9	6.7	10.8	10.6
Total number of leaves differentiated	9.0	8.6	9.0	7.0	10.7	8.9	13.2	12.2	11.8	11.8
Plant height in. cm. ...	9.1	7.1	52.1	24.3	7.4	5.3	20.2	11.8	67.4	54.3
Stem length in cm. ...	0.94	1.17	32.3	15.7	0.39	0.29	2.6	1.6	44.5	32.0
Length of growing point in cm.	0.12	0.12	6.1	2.6	6.03	0.01	1.28	1.06	1.64	1.02
Number of double ridges	13.2	7.9	19.9	13.1	—	—	forming	—	22.4	23.2

Spikelets differentiating in some.

¹ C = Control.

² Deformities in leaves and spikes noted in 8 out of 10 plants.

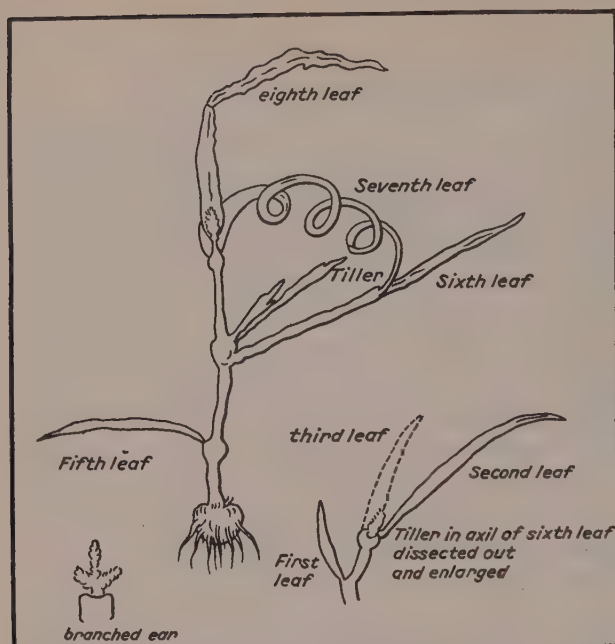


Fig. 1. Morphological deformities in a wheat plant of variety NP 165 due to 2,4-D in the culture medium.

unfolded. The seventh leaf was contorted and its tip was still enclosed in the sheath of the sixth leaf. The lamina of the sixth leaf was also not fully unfolded and it appeared as if its sheath was not wide enough to accommodate the leaf and internode above it and therefore the latter separated out during growth from the sheath. There was also a tiller in the axil of the sixth leaf. The first leaf of this tiller was not fully differentiated into lamina and sheath and it did not completely envelope the internode above it. The second leaf was not fully unfolded, and its sheath was completely closed. The third leaf was enclosed within the second leaf. The internode bore an ear with 9 spikelets. The fifth leaf was more or less normal in shape although it was not fully unfolded.

There was a swelling at the junction of the roots and the shoot, and the roots were short, stubby and often discoloured.

A larger tiller at the base of the plant when dissected out showed an ear with 4 branches, a larger central one about 3 mm long and three smaller ones at its base. The ears that emerged never showed such a peculiar construction although they were often irregular in shape.

The plants of the variety S 40 were affected more or less to the same extent by 2,4-D during the first 6 weeks, but later on the difference between the control and the treated plants diminished. None of the plants showed

abnormalities in morphological differentiation as was noticed without exception in NP 165. Evidently the variety S 40 appeared to be relatively more resistant than NP 165 to 2,4-D, at least at the concentration of 0.25 p.p.m.

Sampling Data (water culture)

The data are listed in tables 2 and 3.

Number of green leaves: When the plants were 6 weeks old (sample 1) the number of fully expanded green leaves per plant was smaller in the treated plants of both varieties, due largely to reduction in tiller number. Thirty days later (sample 2), the leaf number was still smaller in the treated plants of NP 165, whereas the difference in the case of S 40 was negligible. (The total number of leaves unfolded was, however, greater in control if the dry leaves are included.) It is interesting to note that at the age of 72 days the number of dry leaves was much less in the treated plants of both the varieties. This difference between the control and treated plants persisted even at the age of 126 days (sample 3) in variety S 40. This delayed senescence of leaves of the treated plants may be due to their slower rate of expansion and reduced rate of export of nitrogen from them. The percentage nitrogen content of green leaves in NP 165 varied from 4.72 at sample 1 to 2.88 at sample 2 in control and from 3.35 % at sample 1 to 3.43 at sample 2 under treatment; in S 40 from 4.68 at sample 1 to 3.33 at sample 2 in control and from 4.70 % to 4.13 in treated plants (cf. Table 5). This reduced rate of export of nitrogen from the leaves would imply that 2,4-D directly arrested meristematic activity, thus reducing the demand for nitrogen.

Root growth: The root system of the treated plants did not elongate further, as judged by the length of the longest root, after the first six weeks. It would appear that the root system of variety S 40 elongated relatively less as compared to that of NP 165, under the influence of 2,4-D.

At sample 1 (42 days after sowing) the root volume of the treated plants of NP 165 was only 47 %, whereas the dry matter was 70 %, of that of control plants. This indicates that the elongation phase of growth was depressed relatively more. The lower water content of the roots of the treated plants also supports this view. At the time of the second sample (72 days after sowing) the root volume of the treated plants was 46 %, and the dry matter 44 %, of that of control plants, which perhaps indicates that the new roots that developed subsequently were more or less of normal type and probably better adapted to 2,4-D.

More or less similar relations appear to hold in the case of S 40. At sample 1 the root volume was 45 % and dry matter 55 % of that of control; at

Table 2. Growth data for NP 165 (water culture). Mean per plant.

Data measured	Sample 1 42 days after sowing		Sample 2 72 days after sowing	
	Control	2,4-D	Control	2,4-D
1. Tiller number	3.33	0.85	4.65	2.33
2. Fully unfolded leaves:				
Green	12.0	5.7	14.9	7.4
Dry	—	—	9.8	0.9
3. Length of the longest root in cm.	18.5	13.0	30.6	12.8
4. Volume of roots in ml.	3.0	1.42	9.5	4.4
5. Fresh weight of roots in gm.	0.0100	0.8120	7.4150	2.6350
6. Fresh weight of shoots (minus fully unfolded green leaves) in gm.	2.1700	1.0800	13.7050	3.9775
7. Fresh weight of green leaves in gm.	1.1567	0.6117	7.4450	1.3875
8. Fresh weight of the whole plant in gm.	5.3367	2.5037	28.5650	8.000
9. Dry weight of roots in gm.	0.0971	0.0687	0.4250	0.1900
10. Dry weight of shoots in gm.	0.2076	0.1373	2.3850	0.6025
11. Dry weight of leaves in gm.	0.1543	0.0965	1.1050	0.2520
12. Dry weight of the whole plant in gm.	0.4590	0.3025	3.9150	1.0445

sample 2, the volume was 50 % and dry matter 55 % of that of controls and at sample 3, the volume was 88 % and the dry matter 109 % of that of control.

Net Assimilation Rate: The assimilatory efficiency of the leaves of the two sets of plants may be compared by calculating the net assimilation rates according to the method suggested by Gregory (1926). The formula for calculating the net assimilation rate is $\frac{W_2 - W_1}{t_2 - t_1} : \frac{A_2 - A_1}{\log_e A_2 - \log_e A_1}$ where

$W_2 - W_1$ indicates the increment in the dry matter of the plant during a certain time interval, t_1 to t_2 , W_1 being the weight at time t_1 and W_2 at time t_2 . A_1 and A_2 represent the leaf areas on the two respective occasions, and the ratio $A_2 - A_1 / \log_e A_2 - \log_e A_1$ gives the mean leaf area during the time interval under study. Williams (1946) has pointed out that this formula is strictly true if the relation between leaf area and plant weight is linear. In our case this relation was approximately linear and therefore the equation was used directly without employing the interpolation method suggested by Williams. The leaf area was not measured but the dry weight of leaf was used as an indirect measure of leaf area. The net assimilation rates thus calculated are given in table 7.

It will be seen that the net assimilation rate was reduced due to treatment considerably more in NP 165, whereas, in S 40 the treatment had little adverse effect or perhaps an accelerating effect in the later stage.

Table 3. *Growth data for S^c 40 (water culture). Mean per plant.*

Data measured	Sample 1		Sample 2		Sample 3	
	Control	2,4-D	Control	2,4-D	Control	2,4-D
1. Tiller number	3.26	1.15	5.00	3.05	3.00	4.20
2. Fully unfolded leaves:						
Green	9.7	5.3	13.0	14.6	9.4	14.2
Dry	—	—	7.2	0.5	22.0	15.2
3. Length of the longest root in cm.	23.2	12.7	38.3	12.0	42.7	12.7
4. Volume of roots in ml.	1.8	0.8	8.2	4.10	25.0	22.0
5. Fresh weight of roots in gm.	1.4966	0.5017	8.75	4.43	19.08	21.86
6. Fresh weight of shoots (minus fully unfolded green leaves) in gm.	1.1100	0.4183	6.9350	3.1650	29.28	22.24
7. Fresh weight of green leaves in gm.	0.7266	0.3900	5.4150	2.000	9.32	11.02
8. Fresh weight of the whole plant in gm.	3.3332	1.3100	21.1000	9.5950	57.68	55.12
9. Dry weight of roots in gm.	0.0804	0.0441	0.5800	0.3175	2.16	2.36
10. Dry weight of shoots in gm.	0.1538	0.0580	1.0550	0.3650	7.42	4.68
11. Dry weight of leaves in gm.	0.0982	0.0519	0.7900	0.2992	2.90	2.38
12. Dry weight of the whole plant in gm.	0.3324	0.1540	2.4250	0.9817	12.48	9.42

Dry matter production and uptake of nitrogen by the plants: In NP 165 the dry matter and total nitrogen content per plant were reduced by 33 and 53 % respectively in the treated plants after growth during the first 42 days (tables 2 and 6). The root volume was, however, reduced as a result of treatment by 53 % and presumably the total uptake of nitrogen decreased due to reduction in the absorption surface. Although the uptake of nitrogen was reduced by 53 %, tiller number was reduced by nearly 75 % (3.33 in control and 0.85 in 2,4-D), which perhaps indicates that 2,4-D (and not deficiency of nitrogen) acted directly in an adverse manner upon tiller differentiation. It may be noted in this connection that, as compared to controls, a relatively higher proportion of nitrogen content was located in the roots of the treated plants due perhaps to its restricted utilization in the shoot. Since the dry matter of the shoots was not reduced to the same extent as the uptake of nitrogen it would appear that carbon assimilation of the treated plants was perhaps not adversely affected. The larger amount of the dry matter per leaf in the treated plants (17.0 mg. per leaf as against 12.8 mg. per leaf in the control) would seem to bear this out and also point to reduced utilization of carbohydrate as a result of restricted meristematic activity.

Between 42 and 72 days the uptake of nitrogen by the treated plants was lower but the percentage nitrogen content was higher. It has already been stated that the net assimilation rate of the treated plants was lower during this period and this would appear to be a sufficient reason to account for their higher percentage nitrogen content. The leaves that developed during

Table 4. *Moisture content as percentage of dry weight (water culture).*

Plant material	Sample 1		Sample 2		Sample 3	
	Control	2,4-D	Control	2,4-D	Control	2,4-D
<i>NP 165</i>						
Roots.....	1970	1082	1644	1287	—	—
Shoot.....	945	686	474	560	—	—
Leaves	649	534	574	451	—	—
Whole plant	1062	728	629	666	—	—
<i>S 40</i>						
Roots.....	1761	1037	1408	1295	783	826
Shoot.....	622	621	557	676	295	375
Leaves	640	651	585	568	239	363
Whole plant	903	751	770	877	362	485

the period were not of normal type and it is quite likely that their photosynthetic activity suffered on this account, although Freeland (1944) observed that 2,4-D, applied as a mist spray at a concentration of 100 p.p.m. caused a decrease in the rate of apparent photosynthesis in beans observed continuously over a period of two to four days.

The rate of uptake of nitrogen per unit root volume was calculated by the formula $\frac{N_2 - N_1}{t_2 - t_1} : \frac{V_2 - V_1}{\log_e V_2 - \log_e V_1}$, where N_2 and N_1 are the total nitrogen contents of the plants and V_1 and V_2 root volumes at 42 and 72 days respectively. The ratio $\frac{V_2 - V_1}{\log_e V_2 - \log_e V_1}$ gives the mean root volume during this period. The rates of absorption of nitrogen thus calculated, worked out as 8.4 mg. per c.c. root volume in the control and 7.9 mg. per c.c. under treatment, which indicate that absorption capacity of the roots was not affected by 2,4-D, provided volume can be regarded as an adequate measure of the absorption surface of the root. It may also be noted that the relative rates of increase in root volume during this period (as calculated by the formula $\log_e V_2 - \log_e V_1 / t_2 - t_1$) were 1.15 and 1.13 for control and treated plants respectively. The shootroot ratios at 72 days were 8.21 and 4.50 for control and treatment, respectively, thus indicating that shoot growth suffered relatively more under treatment between 42 and 72 days. It would appear that the root system becomes better adapted in course of time to 2,4-D, whereas the shoot growth suffers increasingly more.

Attention may also be called to reduced hydration of the tissues of the treated plants (cf. table 4). The difference persists at the time of sample 2 although it is negligible on the whole plant basis. Currier (1949) also observes that most of the cell responses to 2,4-D and other herbicides are hydration

Table 5. *Nitrogen content as percentage of dry weight (water culture).*

Plant Material	NP 165		S 40	
	Control	2,4-D	Control	2,4-D
<i>Sample 1</i>				
Roots	3.85	3.58	4.25	3.48
Shoot	4.33	2.77	4.07	3.22
Green leaves	4.72	3.35	4.68	4.70
Whole plant	4.30	3.23	4.33	3.80
<i>Sample 2</i>				
Roots	2.49	3.53	1.59	2.77
Shoot	1.05	2.46	2.04	3.32
Green leaves	2.88	3.43	3.33	4.13
Whole plant	2.14	3.14	2.32	3.41
<i>Sample 3</i>				
Roots	—	—	1.69	1.71
Shoot	—	—	0.77	1.18
Green leaves	—	—	1.43	2.76
Whole plant	—	—	1.63	1.88

phenomena involving an increase or decrease in the water content of the cytoplasm.

In S 40 the dry matter and total nitrogen content per plant were lower by 54 and 59 % respectively, in the treated plants, at the end of six weeks (table 3 and 6). The root volume was reduced by 55 % and it would seem that, as in the case of NP 165, the uptake of nitrogen decreased due to reduction in the absorption surface. The uptake of nitrogen was reduced by 59 % and tiller number was reduced only to a slightly greater extent, viz., by 65 %. Apparently 2,4-D did not act so adversely upon tiller differentiation as it did in the case of NP 165. It may also be recalled in this connection that in NP 165 the number of tillers plus tiller buds was 6.4 in the control and 2.8 under treatment, whereas in S 40 it was 5.3 in the control and 4.7 under treatment. Thus practically the same number of tiller initials was laid down in S 40, although their growth was checked. Another point of difference between the two varieties consisted in that, in S 40, the dry matter content per leaf was almost identical in treated and control plants and the percentage content of nitrogen was only slightly less in the former. Evidently growth was largely checked in the treated plants by the decrease in nitrogen uptake as a result of reduction in root growth.

Between 42 and 72 days the uptake of nitrogen in control was 42.8 mg. as against 27.4 mg. under treatment. The percentage nitrogen content was, however, higher in the latter case.

The relative rate of increase in root dry weight was more or less identical in the two cases but the relative rate of increase in shoot dry weight was somewhat less under treatment. The rate of uptake of nitrogen per c.c. root

Table 6. *Absolute nitrogen content in mg. per plant (water culture).*

Plant material	NP 165		S 40	
	Control	2,4-D	Control	2,4-D
<i>Sample 1</i>				
Roots	3.73	2.46	3.42	1.54
Shoot	8.99	3.80	6.27	1.87
Green leaves	7.28	3.23	4.60	2.44
Whole plant	20.00	9.49	14.29	5.85
<i>Sample 2</i>				
Roots	10.58	6.71	9.28	8.79
Shoot	25.05	14.82	21.52	12.12
Green leaves	31.87	8.65	26.33	12.35
Whole plant	67.50	30.18	57.13	33.26
<i>Sample 3</i>				
Roots	—	—	36.50	40.36
Shoot	—	—	57.14	55.04
Leaves	—	—	41.42	65.64
Whole plant	—	—	135.06	161.04

Table 7. *Net assimilation rate in gm. per gm. dry weight of leaf.*

Plant material	Control	2,4-D
NP 165 Between 42 and 72 days	7.00	4.54
S 40 » » » » »	6.30	5.90
S 40 Between 72 and 126 days	6.20	8.40

volume was 10 mg. in control and 13.3 mg. in treated plants. It is significant that as compared to control plants a relatively larger proportion of the nitrogen content of the treated plants was located in the roots. Since the net assimilation rate was not reduced appreciably by the treatment it may be concluded that the higher percentage nitrogen content of the plant was due mainly to enhanced capacity of the roots to absorb nitrogen.

It is significant that the water content of tissues, except that of roots, was not affected by the treatment at sample 1 and no deformities of any sort occurred in this variety (table 4).

Between 72 and 126 days the difference in growth between the control and treated plants diminished still further, due no doubt to the increasing adaptation of the plants to 2,4-D as seen from the course of the various physiological functions.

To sum up: Both NP 165 and S 40 were affected adversely by 2,4-D in the early stages but the latter variety had a better capacity for adaptation than the former and its meristematic tissues were also less severely affected by it. The cause of irregular growth pattern in NP 165 cannot obviously be sought in the derangement of functions like nitrogen absorption or photo-

Table 8. *Grain yield and ear characters. Mean per plant.*

Data	Water culture				Soil culture			
	NP 615		S 40		NP 165		S 40	
	Control	2,4-D	Control	2,4-D	Control	2,4-D	Control	2,4-D
Days from sowing to ear emergence	95.5	100.6	—	—	85.8	93.1*	131.3	130.7
Days from sowing to anthesis	100.6	107.7	—	—	89.3	96.1*	130.9	130.0
Ear number	4.6	1.6	2.4	1.5	5.9	4.4*	4.0	3.8
Spikelet number per ear ...	18.1	10.0	20.5	21.6	16.4	16.7	20.9	20.9
Grain number per ear	17.4	17.8	—	—	29.9	26.8	34.4	28.7
1000-grain weight in gm. ...	25.9	30.0	—	—	44.9	40.7	47.4	45.5
Grain yield in gm.	2.06	0.87	—	—	7.94	4.84*	6.52	5.59

The data from soil culture alone could be subjected to statistical analysis (analysis of variance).

* Significantly different from control at 5 % probability.

synthesis but whether its relation to tissue hydration is causal or spurious would appear worth examining.

Grain yield: The mean number of days taken for ear emergence in the control plants of variety NP 165 was 95 days (from the day of sowing), whereas in the treated plants it was 103 days. Anthesis was noticed on an average after 100 days from sowing in the control plants and after 108 days in the treated plants. The data in table 8 show that ear number, ear length, spikelet number and grain yield were all reduced due to treatment. The absence of any effect of 2,4-D on grain number per ear and 1000-grain weight may perhaps be accounted for by the fact that the contribution of the main shoot was proportionately greater in the treated plants.

The plants of variety S 40 dried up later due to root-rot and no grains could, therefore, be collected. The data on ear number and spikelet number per ear, however, indicate that 2,4-D had perhaps little effect in this variety.

Soil culture

The course of tillering and of increase in height of, and leaf number on, the main shoot is shown in figure 2. 2,4-D had a distinctly adverse effect on tillering in NP 165, as was noticed under water culture. The rate of unfolding of leaves on the main shoot was also slower in the treated plants but the reduction in the total number of leaves on the main shoot was smaller as compared to that in the number of tillers. A similar difference was also noticed in the water culture. Height of the main shoot was also reduced due to 2,4-D. In S 40, 2,4-D had more or less similar effects

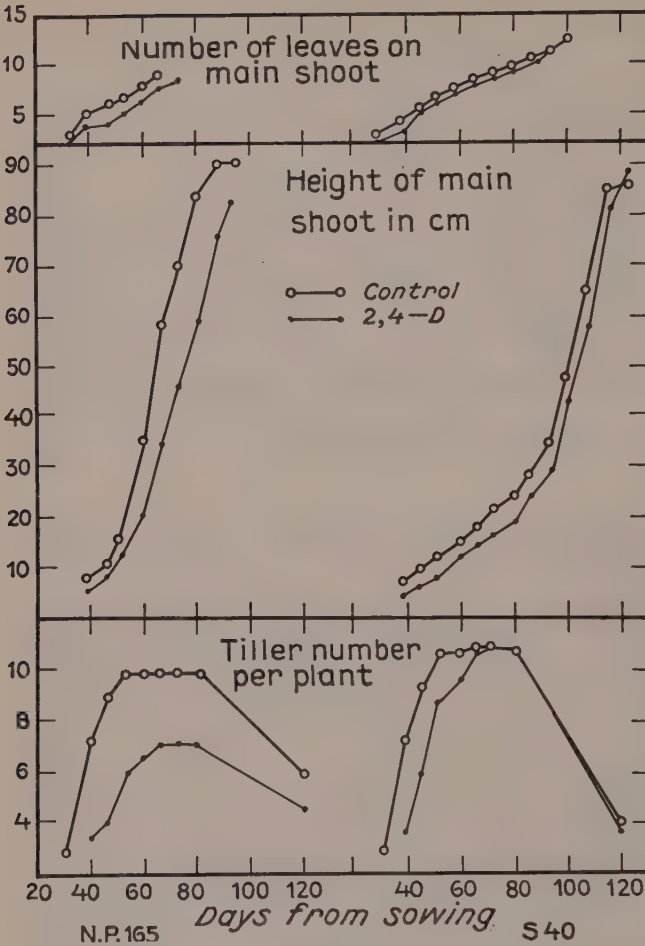


Fig. 2. Effect of 2,4-D on the growth of wheat varieties NP 165 and S 40 grown in soil culture.

in the early stages but later the treated plants made up in growth as was observed under water-culture.

An important difference between the plants of variety NP 165 grown in water and soil culture respectively was that morphological abnormalities so abundantly present in the former were only occasionally noticed in the latter. This was very probably due to the actual concentration of 2,4-D gradually diminishing with time in the soil. No morphological peculiarities were noticed in the treated plants of S 40.

Both ear emergence and anthesis in NP 165 occurred about a week later in the treated plants (table 8). There was no such difference in the case of S 40. It may be noted that as compared to soil culture ear emergence was

delayed in control as well as treated plants of both varieties under water-culture.

Grain yield was reduced by 39 %, due to treatment, in NP 165 and this was largely due to reduction in ear number and to an extent due to reduction in grain number per ear (although this reduction was statistically not significant). Although grain yield of S 40 was lower under 2,4-D, the difference did not reach significance at 5 % probability. In this case the reduction in grain yield was largely due to smaller grain number per ear (although the difference was not statistically significant).

Discussion

2,4-D and nitrogen uptake: Schuffelen (1948) observed that at concentrations between 10^{-16} and 10^{-6} mol/litre naphthalene- and indole-acetic acid had no influence upon the rate of absorption of potassium and sodium during an 8-hour period, by excised roots of oats (Hoagland's technique), but at concentration beyond 10^{-6} mol/litre (equivalent to 0.175 p.p.m. of indole-acetic acid) they depressed the rate appreciably. He believes that the action of the growth substance is not a direct one, but that its high concentration condenses the plasma and thus sugar transport is impeded, the pump function is hampered and ion absorption decreased. From the present long-duration experiment (in which growth continually occurred and hence is not strictly comparable with Schuffelen's) it appears that 2,4-D first interferes with root growth and as a consequence the total uptake of nitrogen, and presumably that of other nutrients, is diminished during the first six weeks, although the rate on volume basis would not seem to be affected. Between 42 and 72 days the relative rate of increase in root volume and the rate of absorption of nitrogen on root volume basis are again similar in both treated and control plants. These results also appear to be at variance with those of Rhodes et al. (1950) in which MCPA (4-chloro 2-methyl phenoxyacetic acid) caused a real reduction in total uptake of potassium, and only a slight one in that of nitrogen and phosphorous, by tomato plants. In their experiment although shoot weight was depressed by MCPA the root weight (and presumably, therefore, the absorption surface) was not affected. They ascribe this effect of MCPA to specific inhibition of potassium uptake by roots, combined with interference with transport from roots to tops or redistribution from tops to roots.

In the present experiment it is not possible to calculate, for want of initial data, the rate of absorption of nitrogen per mean dry weight of roots during the first six weeks, but comparison of nitrogen uptake and root dry weights,

in both the varieties, indicates that the former was reduced relatively much more than the latter by 2,4-D. Between 42 and 72 days the uptake of nitrogen per mean dry weight of roots was reduced by 2,4-D in NP 165 and increased in S 40 (NP 165; 214 mg. in control, 174 mg. in 2,4-D; S 40: 169 mg. in control, 197 mg. in 2,4-D). The question about the correct basis of expressing absorption rate, however, still remains unsolved and further discussion, in absence of more adequate data, would seem unprofitable.

Whether the total uptake of nitrogen was lowered by 2,4-D due to its effect on growth or absorption capacity of roots, it is clear that tiller production was depressed to a relatively greater extent during the first six weeks and hence the inference is perhaps warranted that 2,4-D acted directly upon meristematic activity of the shoot. Rhodes et al (1950) also conclude that the symptoms induced by MCPA were clearly not due to a simple deficiency of potassium in tops. Rasmussen (1947) concludes that although 2,4-D principally destroys carbohydrate reserves in dandelion — much of the loss being due to respiration — the lethal action is due to protoplasmic toxicity. According to him toxicity is directed towards certain protoplasmic compounds, probably proteins, which vary between families and to a lesser extent between genera and species. In view of this suggestion it may be surmised that since S 40 is relatively more resistant to 2,4-D than NP 165 its cytoplasmic proteins may be qualitatively different from those of the latter.

2,4-D and flowering: Since high tiller production and delayed flowering can be reversed to sparse tillering and early flowering by means of long days, it is legitimate to inquire if the same effect cannot be accomplished by a substance which depresses tillering. 2,4-D at the concentration used, however, reduced tillering and ear size and also delayed the emergence of ear in NP 165. Even at the age of six weeks, when no irregularity in growth was apparent, tiller number as well as the number of double ridges was reduced by 2,4-D. So far as can be judged from these data, 2,4-D did not in any way influence the time of the onset of the reproductive phase (as marked by the double ridges) and the delayed emergence of ears in the treated plants was probably due to its depressing effect on stem growth. It is quite conceivable that a smaller (and less toxic) concentration might perhaps have had a different effect. From the soil culture experiment in which the adverse effect of 2,4-D on tillering was of a much lower order (27 % reduction in ear number as against 64 % under water culture) it is, however, seen that there was no such expected effect, nor was the number of spikelets increased as reported by Leopold and Thimann (1948), who investigated the effect of naphthalene and indole-acetic acid. In the late variety, S 40, 2,4-D also did not influence the onset of the reproductive phase, ear emergence or spikelet number, although tiller production con-

tinued at a lower level during the initiation of the reproductive phase. Thus these observations do not appear to be in agreement with those of Leopold (1949) who investigated the interaction of day-length and auxin (naphthalene-acetic acid) application on tillering in wintex barley, which flowers under a day-length of 16 hours and only tillers (but probably does not flower or flowers late) under a tenhour day. On feeding auxin through leaves to plants growing under 16- and 10-hour day-lengths, respectively, tillering was completely suppressed under the former day-length but hardly at all under the latter. His inference from this observation that under a long day tillering is suppressed due to higher auxin production does not appear, however, to be entirely acceptable. Leopold and Thimann (1948) also observed that low concentrations of auxin, which suppressed tillering, definitely increased the number of flower primordia and inferred that auxin may not be acting simply in opposition to flowering. Common observation however indicates that the main effect of long day consists in reducing tillering, ear size (number of spikelets) and also the time of ear emergence, and it would, therefore, appear difficult to reconcile this effect of long day with the one supposed by Leopold and Thimann to stimulate auxin production and to be equivalent to increasing the number of flower primordia by an external supply of growth substance.

2,4-D and tissue hydration: That 2,4-D did not induce any irregular growth in S 40 has already been referred to and the question was raised whether this had any relation to tissue hydration. Northen (1942) has observed that application of growth substances like indole-3-acetic acid, indole-3-propionic acid and naphthalene acetic acid, decreased the structural viscosity of protoplasm which probably resulted from dissociation of cellular proteins. According to him the dissociated proteins may be rearranged (reassociated) to form a dissimilar pattern, one that will function differently. Northern (1943) also observed that incipient drought conditioned a decrease in the structural viscosity as a consequence of protein dissociation and suggested that such a dissociation may condition an increased protoplasmic swelling pressure, accelerated rates of respiration and polysaccharide hydrolysis and may at times lead to mitotic abnormalities.

In view of the similarity in some of the effects of growth substances and incipient drought, as suggested by Northen, it may be inquired whether a variety like S 40, in view of its relatively greater resistance to the action of 2,4-D, would not prove relatively more drought-resistant than NP 165. The problem of drought-resistance is no doubt very complex and its basis must necessarily depend on the objective in view. From agronomic point of view, for instance, a variety that yields relatively better under restricted soil-moisture supply may be regarded as drought-resistant. A preliminary experi-

ment under pot-culture, on S 40 and NP 165, with normal water supply and intermittent drought (reduced frequency of watering), gave some indication of S 40 being a relatively better yielder under drought. Comparison of these two varieties was, however, complicated by the fact that ear emergence in S 40 could only be synchronized with that in the other variety by means of extra hours of light with a consequent reduction in tillering and ear size in the former. For the present, it can only be hoped that if a correlation between resistance to 2,4-D (absence of irregular mode of growth) and a superior yielding capacity under restricted moisture could be established, it might serve as a useful index for comparing varietal drought-resistance in the seedling stage.

Summary

The results of an investigation upon the effect of 2,4-D in the culture medium on the growth and development of two varieties of wheat are presented. The plants were raised both in water and soil culture (in pots) and the strength of 2,4-D was maintained at 0.25 p.p.m. in the former and about 1 p.p.m. in the latter. The effective concentration must have been, however, much lower in the soil in view of its gradual decomposition and as could be judged from the effects obtained. The varieties used were NP 165, an early vulgare type and S 40, a late durum.

Plant samples were collected from the water culture on the 42nd and 72nd day, respectively, after sowing and the dry matter of the different organs as well as their nitrogen content was determined. A third sample of variety S 40 was also taken on the 136th day after sowing. Plants were also dissected to study the developmental stage of the plants on these occasions.

Dissection at the age of six weeks revealed that the final number of leaves on the main shoot of NP 165, about 9, was already laid down and that 2,4-D had little effect. It had, however, a significantly depressing effect on the differentiation of tillers (tillers plus tiller buds), the number being 6.4 in the control and 2.8 under 2,4-D and of spikelet primordia. Between 42 and 72 days there also appeared various deformities such as irregular unfolding of leaves, incomplete differentiation of lamina and sheath, twisted and occasionally branched ears, swelling at the junction of roots and shoot, and short, stubby and often discoloured roots. No morphological deformities were noticed in the variety S 40.

The net assimilation rate (between 42 and 72 days) was reduced due to 2,4-D, only in variety NP 165 and this is attributed to the abnormal pattern of leaves.

In NP 165, during the first 42 days, both the shoot and root dry weight decreased due to treatment and the total nitrogen content of the treated

plants was reduced relatively more than the plant dry weight but more or less in proportion to root volume. Apparently nitrogen uptake was lowered primarily due to reduced absorption surface. Since tiller differentiation was reduced relatively more than the uptake of nitrogen it is surmised that 2,4-D directly influenced meristematic activity. Between 42 and 72 days the uptake of nitrogen by the treated plants was lower but the percentage nitrogen content was higher, which may perhaps be accounted for by the lower net assimilation rate during this period. The rate of uptake of nitrogen per unit root volume was not affected by 2,4-D, which again indicated that the uptake of nitrogen was lower due to restricted root growth and not due to diminished absorption capacity.

The lower nitrogen uptake by the treated plants of S 40, during the first six weeks, was mainly due to decrease in root growth and perhaps slightly due to reduced absorption capacity. The decrease in tiller production was slightly greater than that in nitrogen uptake. As the effect on the differentiation of tillers (tillers plus tiller buds) was very slight, it would appear that diminution in the further growth of tillers was largely due to reduced nitrogen uptake and in a small measure due to the effect of 2,4-D. Between 42 and 72 days, the rate of uptake of nitrogen, on the basis of volume as well as dry weight of roots, was higher in the treated plants and thus the higher percentage nitrogen content of the treated plants may be accounted for. After 72 days the treated plants improved in growth and ultimately came up to the level of control plants.

The growth in soil culture followed more or less the same course as under water culture. Morphological deformities of only a minor degree were noticed in NP 165 alone. Grain yield of the treated plants was reduced only in NP 165 and this was due to decrease in ear number.

In spite of depression in tillering due to 2,4-D, neither was the onset of the reproductive phase nor the emergence of the ears hastened in any variety. The number of spikelets per ear was reduced due to 2,4-D in NP 165 only under water culture.

It is not clear from the available data whether the relatively smaller effect of 2,4-D on the water content of the tissues of S 40 had any causal connexion with its freedom from morphological deformities. In view of the similarity in some of the effects of growth substances and incipient drought, as observed by Northen (1943), it is suggested that if a correlation between resistance to 2,4-D (absence of irregular growth pattern) and a superior yielding capacity under restricted water supply could be established, it might serve as a useful index for comparing varietal drought-resistance in the seedling stage.

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References

- Åberg, E. & Denward, T.: Abnormal spikes in Barley caused by hormone derivatives. — Ann. Roy. Agric. Coll. Sweden, 14: 366. 1947.
- Clark, E. & Wittwer, S. H.: Effect of certain growth regulators on seed stalk development in lettuce and celery. — Plant Physiol. 24: 555. 1949.
- Currier, H. B.: Response of plant cells to herbicides. — Ibid. 24: 601. 1949.
- Freeland, R. O.: Effects of growth substances on photosynthesis. — Ibid. 24: 621. 1949.
- Gregory, F. G.: The effect of climatic conditions on the growth of barley. — Ann. Bot. 49: 1. 1926.
- Hoagland, D. R. & Arnon, D. I.: The water culture method for growing plants without soil. — Univ. Calif. Agr. Exp. Sta., Circ. 347: 1. 1938.
- Kaiser, S. & Albaum, H. G.: Early root and shoot growth in two varieties of *Avena sativa* in relation to growth substances. — Am. J. Bot. 26: 749. 1939.
- Leopold, A. C. & Thimann, K. V.: Auxin and flower initiation. — Science 108: 664. 1948.
- Leopold, A. C.: The controlling of tillering in grasses by auxins. — Am. J. Bot. 36: 437. 1949.
- Northern, H. T.: Relation of dissociation of cellular proteins by auxins to growth. — Bot. Gaz. 103: 668. 1942.
- Relationship of dissociations of cellular proteins by incipient drought to physiological processes. — Ibid. 104: 480. 1943.
- Rasmussen, L. W.: The physiological action of 2,4-Dichlorophenoxyacetic acid on Dandelion, *Taraxacum officinale*. — Plant Physiol. 22: 377. 1947.
- Rhodes, A., Templeman, W. G., & Thruston, M. N.: The effect of the plant growth regulator, 4-chloro 2-methyl phenoxyacetic acid, on the mineral and nitrogen contents of plants. — Ann. Bot. N.S. 14: 181. 1950.
- Rossmann, E. C. & Sprague, G. F.: Effect of 2,4-D on yields of maize in the succeeding generation after treatment. — Plant Physiol. 24: 770. 1949.
- Rossmann, E. C. & Staniforth, D. W.: Effects of 2,4-D on inbred lines and a single cross of Maize. — Ibid. 24: 60. 1949.
- Schuffelen, A. C.: Growth substances and ion absorption. — Plant and Soil 1: 121. 1948.
- Williams, R. F.: The physiology of plant growth with special reference to the concept of the net assimilation rate. — Ann. Bot., N.S. 10: 41. 1946.
- Zimmerman, P. W.: Present status of »Plant Hormones». — Industr. Eng. Chem. 35: 596. 1943.

Inorganic Carbon Sources of Green Algae. I. Growth Experiments with *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*

By

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In a previous paper (Österlind 1949, Fig. 20) it has been shown that the growth rate of *Scenedesmus quadricauda* in nutrient solutions, aerated with air, is rather slight at pH below 5.5. Then it rises steeply and at pH 6.5 it is near its optimum value. This was assumed to depend on the increasing concentration of bicarbonate ions. It was shown that the growth rate was directly proportional to the concentration of bicarbonate ions below 10 $\mu\text{mol/l}$.

The correctness of this interpretation was doubted by Steemann Nielsen & Kristiansen (1949) (also personal communication). According to them, the high sensitivity of the growth to variations in pH in the interval 5.5–6.5 would be a direct effect of pH. The rapid growth at pH 6.5, for example, would then be due only to CO_2 , the concentration of which is 10 $\mu\text{mol/l}$.

Scenedesmus quadricauda

To obtain further data about these questions, the growth experiments with *Scenedesmus quadricauda* at various pH values have been extended also to CO_2 concentrations lower than that of usual air (0.03 %).

The same strain was used as previously. The inoculating material was grown for 4–5 days in stoppered flasks with 1 litre of nutrient solution C (Österlind 1949, p. 41), containing carbonate to which HCl was added to the bicarbonate point. In the experiments the phosphate buffered nutrient solution D was used, but 10 $\mu\text{g Zn/l}$ was added. This addition had no effect on growth. pH was regulated by addition

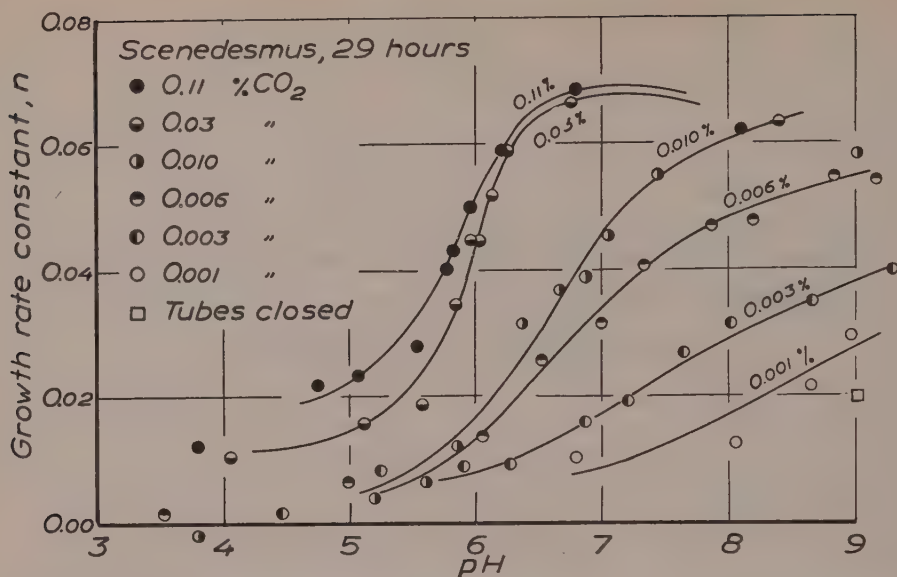


Fig. 1. Growth rate of *Scenedesmus quadricauda* during 29 hours at various pH values in nutrient solutions, aerated with various CO₂ concentrations.

of HCl. Usually 10 pH values were used in each experiment. At each pH value 10 ml were distributed to each of four tubes. One tube was used for pH determination after equilibrium in the dark had been reached with the aerating gas (usually after 20 hours). The tubes were placed in a vertical position around a water-cooled 1,000-watt incandescent lamp. The intensity of light was 6,000 lux. At this intensity the optimum growth rate was reached except during the first 3 hours when an increasing growth rate was obtained at least up to 12,000 lux. Growth was measured photometrically in unfiltered light from an incandescent lamp. Growth rate was calculated as described previously.

In Fig. 1 are shown the curves from experiments at various CO₂ concentrations in the aerating gas. The curves are displaced towards higher pH values at lower CO₂ concentrations. Below 0.01 % of CO₂ the highest growth rate is not reached even in the most alkaline solutions.

Fig. 2 shows theoretical curves for the various CO₂ concentrations, these curves having been calculated in the following way. Of the curves in Fig. 1 that of 0.03 % of CO₂ is best known, and it has previously been shown that the same curve is obtained when there is no depletion of CO₂ or HCO₃⁻ from the nutrient solution. From three experiments at 0.03 % of CO₂ the growth rates have been determined at various HCO₃⁻ concentrations. A straight line has been drawn through these points (cf. Österlind 1949, Fig. 32), and from this line the curve of 0.03 % in Fig. 2 has been calculated. The

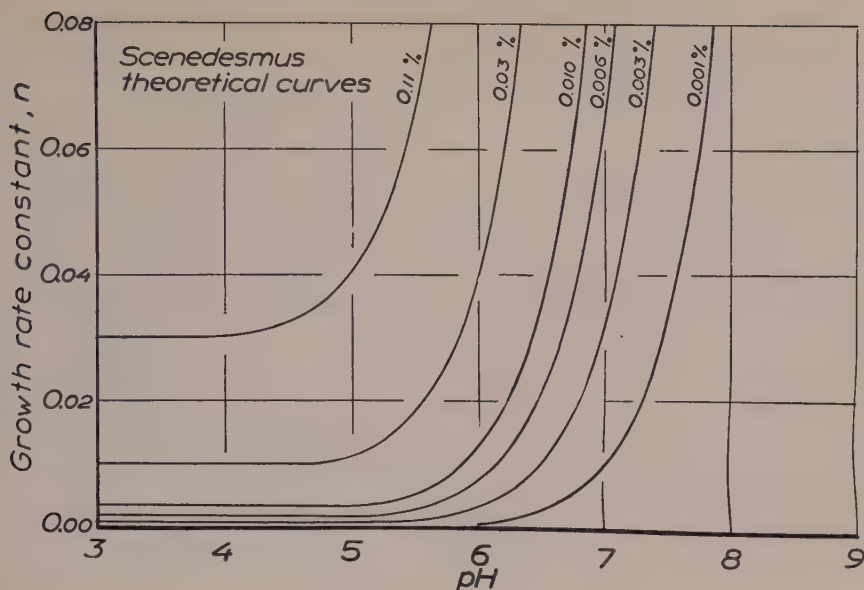


Fig. 2. Theoretical growth curves at various pH values in nutrient solutions, aerated with various CO_2 concentrations.

corresponding curves of the other CO_2 concentrations were then calculated on the assumption that the growth rate depends only on the CO_2 and HCO_3^- concentrations. A comparison between the experimental curves (Fig. 1) and these theoretical curves (Fig. 2) shows, however, great differences even taking into account the fact that no factors which limit growth at high growth rates have been considered. Thus, the curve of 0.11 % of CO_2 is displaced to the right. This agrees with the previously obtained curves (Österlind 1949, Fig. 31) and caused the weak inclination of the 0.12 % curve in Fig. 37. The curves of lower % of CO_2 than 0.03 show a weaker inclination than the theoretical ones.

Fig. 3 shows the growth rates in the same experiments as in Fig. 1 but after only 5 hours illumination. In spite of the great uncertainty of measurements of the small growth values after so short a time, it may be stated that these curves run in another way than after 29 hours. The curve of 0.11 % agrees better with the theoretical curve, at least in its lower parts. At the lower CO_2 concentrations it may be stated that the inclination of the curves is much steeper and more in accordance with the theoretical curves after 5 than after 29 hours. Even at the lowest concentration, 0.001 % of CO_2 , almost full growth rate has been reached at the highest pH value. The reason why the rapid growth cannot be maintained at long illumination must

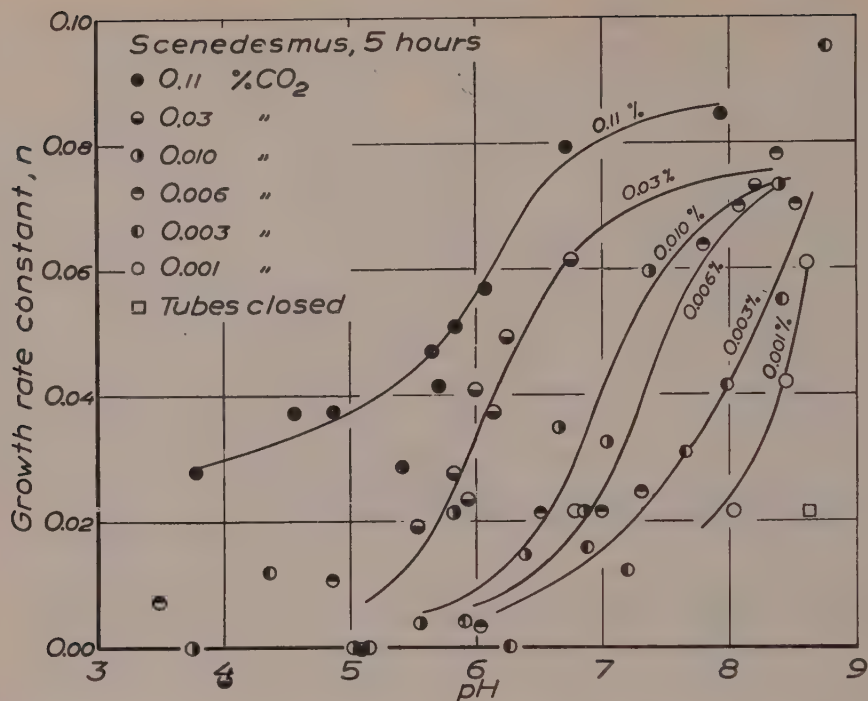


Fig. 3. Growth rate of *Scenedesmus quadricauda* during the first 5 hours at various pH values in nutrient solutions, aerated with various CO₂ concentrations.

be that the inorganic carbon source does not have time to be compensated for by the small amount of CO₂ in the aerating gas.

To examine the content of inorganic carbon sources in the inoculating material a nutrient solution was boiled at low pH to expel CO₂, cooled under soda lime, and solid phosphate was added in the same amount as is usually added from a stock solution. The solution was inoculated and distributed to the tubes as rapidly as possible. The tubes were closed with rubber stoppers and sealed with paraffin. A small glass pendulum in each tube provided an effective means of stirring the solutions when the tubes were continuously shaken. Growth in these solutions, which should contain as little bicarbonate as possible, is indicated by a square in the figures. The site of this point shows that the rapid growth at high pH values and low CO₂ concentration in the gas cannot be due to internal bicarbonate sources.

On account of the slow progress of CO₂ from the gas phase to the liquid phase and further to hydrated form, the curves from low CO₂ concentrations may not be used for calculation of the growth rate at various bicarbonate

concentrations. The consumption of bicarbonate by the algae is so high that the real bicarbonate concentration is lower than the theoretically calculated equilibrium concentration. It has previously been shown that such factors do not influence the 0.03 % curve.

Until determinations of the rate of photosynthesis at bicarbonate concentrations below 10 $\mu\text{mol/l}$ have been made, it is therefore impossible to state with certainty the influence of these low concentrations. It must, however, be stated that bicarbonate is really used by *Scenedesmus quadricauda*. If not, one must assume that as low a concentration as 0.3 $\mu\text{mol CO}_2/\text{l}$ (0.001 % of CO_2 in air) permits full growth of the alga, and this seems rather improbable.

It may be added that an experiment with 0.05 % Ca/l , added to the carbonate buffered nutrient solution, gave exactly the same curve in 0.03 % of CO_2 as with the usual 5 mg Ca/l . This argues against the theory that the curve would be due to a direct pH effect, as Ca usually has an inhibiting effect on the injuries on growth, caused by low pH.

Chlorella pyrenoidosa

The genera *Scenedesmus* and *Chlorella* differ in several physiological properties (e.g. Gaffron 1940, Alg  us 1948). Therefore it would be of interest also to compare the two genera with respect to bicarbonate assimilation.

A strain of *Chlorella pyrenoidosa*, isolated as a bacteria free single cell culture by Dr. Wilhelm Rodhe, was used. The inoculation material was taken from a 4 days old culture in nutrient solution C (carbonate buffered, without HCl) (  sterlind 1949, p. 41), aerated with about 2 % of CO_2 in air. The previously used method of growing the algae in tubes during the experiment was impossible. Due to the production of surface active substances (Pratt 1948) the cultures foamed when aerated and the cells fastened to the walls of the tubes. Therefore the experiments were performed in 100 ml conical flasks, each flask containing 50 ml of nutrient solution. The aerating glass tube was placed in the centre of the flask and thus the air bubbles were broken before they reached the walls of the vessel. The flasks were illuminated from below by a 45   mirror. The same light source was used as previously, and also the same light intensity. The effective intensity inside the culture vessels is, however, certainly a little higher in the flasks than in the previously used tubes. Growth was measured photometrically by pouring about 10 ml into a test tube. At each pH value two flasks were used, and 6 or 8 pH values could be examined in the same experiment.

Figs. 4 and 5 show the result of these experiments with *Chlorella pyrenoidosa* after 5 and 29 hours. It is seen that in cultures, aerated with usual air (0.03 % of CO_2), there is very slow growth at all pH values. In 0.1 % of

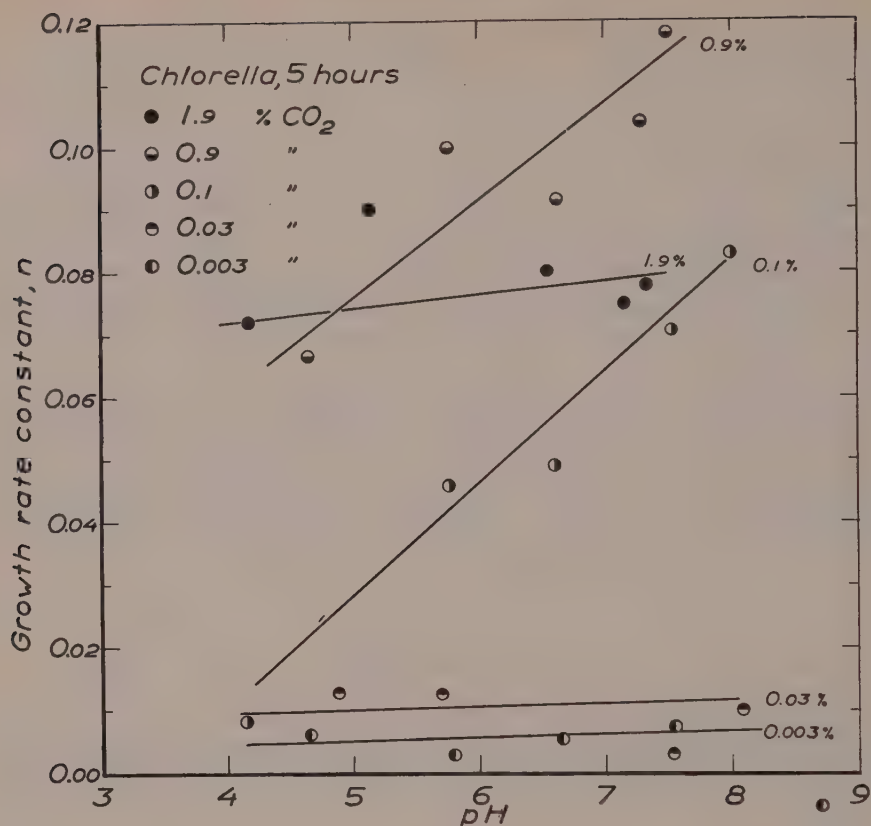


Fig. 4. Growth rate of *Chlorella pyrenoidosa* during the first 5 hours at various pH values in nutrient solutions, aerated with various CO_2 concentrations.

CO_2 good growth occurs at high pH values, falling linearly with decreasing pH. When the CO_2 concentration is increased to about 1 % there is good growth at all pH values between 4 and 8.

The inclination of the 0.1 % curve has nothing to do with the bicarbonate concentration, as there should then be good growth at the highest pH values of the 0.03 % curve. Thus it must be concluded that the *Chlorella*-strain used has no possibility to use bicarbonate at all. In this respect it seems to agree with the water-moss *Fontinalis* (Steemann Nielsen 1947, Ruttner 1947).

The difference between *Chlorella* and *Scenedesmus* has nothing to do with the fact that the inoculating material of *Scenedesmus* was grown in a closed vessel with carbonate-hydrochloric acid, whereas *Chlorella* was grown in a carbonate buffer, aerated with 2 % of CO_2 . In a special experiment, *Scenedesmus* was grown in the same way as *Chlorella* (inoculating material grown

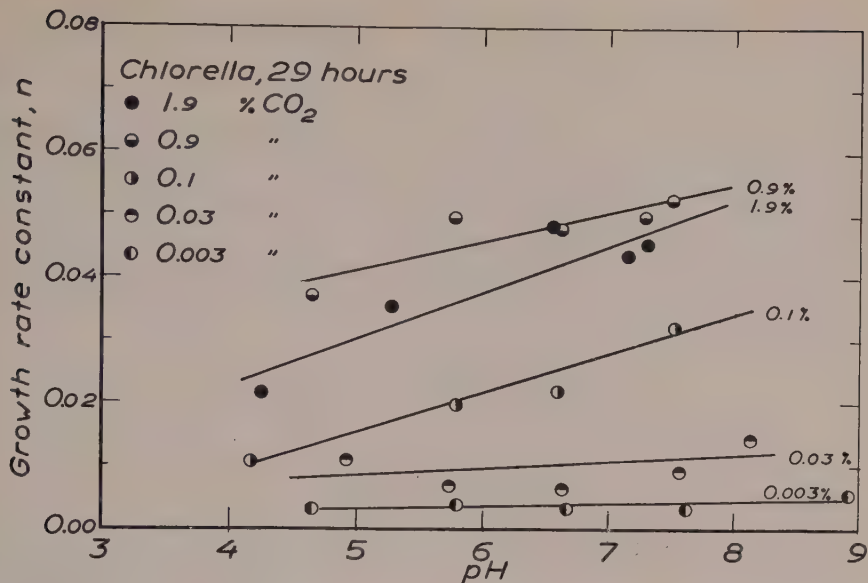


Fig. 5. Growth rate of *Chlorella pyrenoidosa* during 29 hours at various pH values in nutrient solutions, aerated with various CO_2 concentrations.

in a carbonate buffer with 2 % of CO_2 and experiment performed in conical flasks instead of in tubes, aerated with 0.03 % of CO_2). Exactly the same curve was obtained as previously.

Summary

There are at least two types of planktic algae with respect to bicarbonate assimilation. The one extreme is represented by *Chlorella pyrenoidosa*, which has no possibility to use bicarbonate. Another type is represented by *Scenedesmus quadricauda*, which assimilates bicarbonate very well. The assimilation of unhydrated CO_2 does not differ significantly in the two types.

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References

- Alg  s, S.: The utilization of glycocoll by *Chlorella vulgaris*. — *Physiol. Plant.*, 1: 236. 1948.
 Gaffron, H.: Carbon dioxide reduction with molecular hydrogen in green algae. — *Amer. Journ. Bot.* 27: 273. 1940.

- Österlind, S.: Growth conditions of the alga *Scenedesmus quadricauda* with special reference to the inorganic carbon sources. — *Symbol. Botan. Upsaliens.*, 10, No. 3. 1949.
- Pratt, R.: Studies on *Chlorella vulgaris*. XI. Relation between surface tension and accumulation of chlorellin. — *Amer. Journ. Bot.* 35: 634. 1948.
- Ruttner, F.: Zur Frage der Karbonatassimilation der Wasserpflanzen. Eine vergleichende Untersuchung. I. Teil: Die beiden Haupttypen der Kohlenstoffaufnahme. — *Österreich. Bot. Zeitschr.*, 94: 265. 1947.
- Steemann Nielsen, E.: Photosynthesis of aquatic plants with special reference to the carbon-sources. — *Dansk Bot. Ark.*, 12 (8): 1. 1947.
- and Kristiansen, J.: Carbonic anhydrase in submersed autotrophic plants. — *Physiol. Plant.*, 2: 325. 1949.

The Action of 6-Aminoundecane on Wheat Seedlings

By

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Introduction

According to Veldstra (6) fatty acids with branched chains have a greater physiologic activity than acids with a straight chain of similar size and structure, which is explained by the balance between the lipo- and hydrophilic groups. Burström's results (1—3) with di-n-amylacetic and lauric acids confirm this view for branched and unbranched lipophilic chains containing a hydrophilic COOH.

For the present investigation Dr Veldstra has kindly provided a preparation of 6-aminoundecane-HCl (6-A.U.), $C_5H_{11} \cdot CH(NH_2) \cdot C_5H_{11}$. This compound and di-n-amylacetic acid, $C_5H_{11} \cdot CH(COOH) \cdot C_5H_{11}$ are structurally very much alike, but in the middle of the chain, in the place of the hydrophilic carboxyl, it contains an amino group.

The aim of the present study was to investigate whether the effect of the amine differs from that of the acid, and in the course of this investigation various physiologic processes, namely growth, transpiration, water balance, and permeability have been studied.

The plant material was spring wheat, and the culture technique employed was essentially the same as in earlier investigations (1—3). The experiments were carried out in climatic chambers with constant ventilation, at a temperature of 25° C, and with fluorescent light lamps. They were continued for 5 days. The basic nutrient solutions were of the following composition: KH_2PO_4 1/1000, $Ca(NO_3)_2$ 1/1000, KCl 1/1000, $MgSO_4$ 1/2000, $FeCl_3$ 1/50000 and $MnSO_4$ 1/50000. The concentration of the amine was up to 10^{-4} mol.

The pH at the beginning of the experiments amounted to 4.8 in the controls, 5.7 in $3 \cdot 10^{-5}$ mol amine solution, and 4.8 in 10^{-4} mol. A shift towards the alkaline side was observed in the course of the experimental period; it amounted in one instance to 1.9 in the controls, 1.2 in $3 \cdot 10^{-5}$ mol, and 1.7 in 10^{-4} mol; thus the pH varied within the limits 4.8 to 6.9.

Main observations



Fig. 1. *The average shape of plants grown for 4 days in 6-A.U. 10^{-4} mol. To the right control, to the left 6-A.U. treated plant.*

The growth of shoots of the amine-treated plants started to differ from that of the controls within the first 2 days. There was then a significant delay in shoot growth in 10^{-4} mol, but there was no evidence of any change in the roots of this time. At the end of the third day both shoots and roots of amine-treated plants were considerably shorter than those of the controls. This continued for the 6 days the plants were followed. Table 1 shows the resultant gradual decrease for both parts of the plants. As is seen from the obtained values the retardation in 10^{-4} mol was more pronounced on the fourth day (figure 1). At this age of the plants a delay was also noticeable in 10^{-5} mol. As is shown in table 1 the differences between the measurements of both the roots and shoots of 6 day old plants and of the controls were statistically very significant. A consistent reduction was obtained also in 10^{-6} mol, so that it seems probable that the amine is active already in this concentration.

The effect of the amine appears earlier in shoots and is pronounced in 10^{-4} mol concentration when the plants are 4 days old. In this stage, brownish spots appear a few centimeters from the tips of the roots. A special characteristic of the action of the amine is that the leaf bases, 15 mm above the caryopsis, start to wilt, and the leaves consequently bend to one side as is seen in figure 2. These symptoms were much more obvious in the 5 day old seedlings. Subsequently the leaves as a conse-

Table 1. *Shoot and root length of plants treated with different concentrations of 6-A.U. Experiment with flowing nutrient solution. Average of 12 plants, lengths in mm of first leaf and three initial roots.*

Concentration	2 days old		4 days old		6 days old	
	shoots	roots	shoots	roots	shoots	roots
0	44.4 \pm 1.2	53.8 \pm 1.5	96.5 \pm 2.3	92.1 \pm 1.7	149.1 \pm 2.0	121.1 \pm 2.5
10^{-6}	42.4 \pm 1.5	49.8 \pm 1.5	93.9 \pm 1.8	88.7 \pm 1.5	142.7 \pm 2.2	116.9 \pm 2.3
10^{-5}	39.0 \pm 1.2	46.7 \pm 1.4	85.7 \pm 2.4	77.4 \pm 2.1	127.0 \pm 2.6	104.4 \pm 2.7
10^{-4}	35.9 \pm 1.2	48.8 \pm 1.4	67.4 \pm 1.7	74.3 \pm 1.5	94.8 \pm 2.9	95.2 \pm 2.1



Fig. 2. *Plants showing incipient wilting after 5 days. To the right control, to the left amine-treated plants. Note the sharp bending of plants near their bases.*

quence of this bending wilt permanently and die. The same phenomenon was also observed with lower concentrations ($3 \cdot 10^{-5}$ mol) but to a lesser degree. In higher concentration such as 10^{-3} mol the compound was completely poisonous.

In order to find the cause of this reaction a detailed study was made of the effect of the amine on leaves and roots with micro- and macroscopic examinations.

Investigations of the wilted leaves

The wilting must be due to changes in the water balance of the plant, either water absorption or transpiration, or migration of water. Therefore, in the first series, with 4 and 5 day old plants, transpiration measurements were carried out by weighing excised leaves under the given, constant standard conditions. For this purpose lots of three leaves, 75×4 mm in size, were cut off from different sets of plants and placed on an analytical torsion balance for the measurements. For the first 10 minutes readings were made each minute, then up to 20 minutes they continued at 5 minute intervals. The result of these series are summarized in table 2.

As the table shows there was practically no difference in the transpiration, although the leaves had started wilting. In the same series of experiments the water content and the water content/dry matter of 5 day old leaves were determined. Table 3 shows the results of this series. As is seen there was a decrease in the water content of the amine-treated leaves. In another series

Table 2. *The transpiration of plants treated with 6-A.U.* Transpiration values for 3 leaves given in mg/20 min. Concentration of 6-A.U. in mol. Average of 5 experiments.

Age of the plants	Addition of 6-A.U.		
	Control	$3 \cdot 10^{-5}$	10^{-4}
4 days	6.9	6.1	6.1
5 days	6.3	6.1	5.4

Table 3. *Water content of 5 day old amine-treated leaves from the transpiration experiments.*

Water content	control	$3 \cdot 10^{-5}$	10^{-4} mol
% of fresh matter	89.82	87.87	87.48
water cont./dry matter	8.82	7.24	6.99

corresponding determinations were made for the shoots and the roots of the whole plant. Table 4 contains the results of these measurements in three concentrations and at three intervals of time.

As in the earlier experiments the action of the amine appears after 4 days and is significant already in the concentration 10^{-5} mol. It must be emphasized that the reduction of the growth as measured by the dry weight appears earlier and at a lower concentration for both shoots and roots than the impaired water content.

Microscopic investigations of such wilted leaf bases showed a characteristic folding at the walls of the chlorenchyma cells (figure 3 A). At the

Table 4. *The influence of 6-aminoundecane on the growth of wheat plants.* Experiment carried out in flowing nutrient solutions. Dry matter in g per 12 plants and water content g per g dry matter.

6-A.U.		Days					
		2		4		6	
		dry matter	water content	dry matter	water content	dry matter	water content
0	root	0.046	10.5	0.071	11.8	0.100	12.3
	shoot	0.053	8.8	0.112	9.4	0.216	8.8
10^{-6}	root	0.048	9.8	0.074	12.5	0.100	12.8
	shoot	0.054	8.6	0.125	9.3	0.210	8.9
10^{-5}	root	0.040	10.5	0.055	11.4	0.082	12.3
	shoot	0.044	9.2	0.093	9.1	0.177	8.1
10^{-4}	root	0.038	10.0	0.062	10.1	0.077	11.7
	shoot	0.037	9.8	0.068	7.7	0.109	5.7

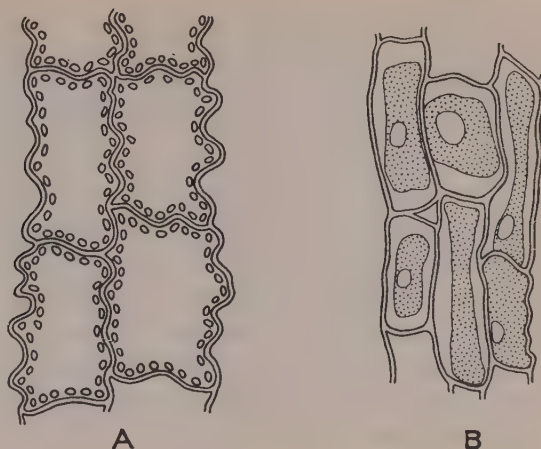


Fig. 3. Cells from the base of the leaf sheath in 6-A.U. treated plants. A, Chlorenchyma cells with folded walls, B, colourless parenchyma from the bundle sheath showing plasmolysis. $\times 28$.

same time the protoplast of the colourless parenchyma from the bundle sheath started contracting, and a real plasmolysis of the cells was observed (figure 3 B). This is reversible, i.e., when the plants were put back with the roots into water under the same thermostatic conditions they resumed their normal turgidity. It is obvious that this localized loss of turgidity of the cells is the cause of the bending.

This phenomenon always seems to appear at a certain stage of development of the leaves. The affected part of the leaf bases seems to correspond to the zone of the most rapid elongation.

Investigations of the roots

A close microscopic examination of the roots has revealed that the brownish spots which appeared on the surface of the roots were caused by the protoplasmic coagulation of the trichoblast cells, and here also the effect of the compound was localized to the elongation zone. In earlier investigations with compounds of the same nature (3), the conclusion was drawn that the elongating cells are especially more sensitive than meristems and mature derivatives. In the present instance this sensitivity is confined particularly to the trichoblast cells of the root which is surprising, because they elongate more slowly than the atrichoblasts.

Effect on the permeability

For a study of the degree of destruction of the organs both root and leaf cells were subjected to a closer microscopical investigations. For this purpose

qualitative permeability measurements were performed by applying staining and plasmolytic methods. For the preservation of the protoplast in an undamaged condition a very dilute solution of neutral red was used in staining the tissues before plasmolysis. The exposure time, determined experimentally, for obtaining a suitable intensity of colour of the cells of amine-treated plants was 15 minutes for the leaves and 1 for the roots. In control plants, the time necessary to give the same effect was much longer than that of the former set. In the same duration of time the leaf cells remained practically colourless and the root cells were very faintly stained. In a second series the time of plasmolysis was measured. Both root and leaf bases of control and treated samples were plasmolysed in 0.40 mol sucrose solution. An exposure time of 15 minutes for cells of amine-treated roots and shoots caused strong plasmolysis, except on the killed trichoblast cells of the roots. In order to obtain the same stage of reaction in the control test a much longer time was necessary, it varied from 40 to 60 minutes. This implies a three to four-fold increase in the rate of plasmolysis under the influence of 6-A.U.

In the cells of shoots and roots deplasmolysis was also studied, but in both parts of the plant the rate was so high that no differences between treated and untreated samples could be obtained. Both plasmolysis and deplasmolysis experiments were carried out in solutions or water without the addition of 6-A.U.

The above mentioned results permit the conclusion that in the cells of roots and shoots in amine-treated plants the permeability both to water and to the dye stuff had increased. In another series of experiments the plants were left in nutrient solutions for 2 days and on the third day they were transferred to solutions containing 10^{-4} mol amine. This was done in order to see how soon or at what stage the permeability changes appeared. Twenty four and 48 hours after the treatment, there was no significant difference between the control and the treated plants, but after about 72 hours the amine-treated ones started to show wilting leaves and the brownish spots on their roots. According to these results the effect of the compound does not develop at once nor at a fixed time after the application of 6-A.U., but it always starts when the leaves are about 5 days old which means that a certain stage of development is especially sensitive.

The effect of the compound on the permeability was tested also according to Veldstra's beet test method (6). The technique employed was essentially the same as that of Veldstra: with a suitable cork borer cylinders were punched out from red beets and sectioned with a double razor blade into pieces 10 mm in diameter and 3 mm thick. The pieces thus prepared were washed for 5 minutes under running tap water in order to clean the cut surfaces. Then they were left for 1 hour

in distilled water which was renewed several times. Finally 10 selected pieces were placed into the experimental solutions of 50 ml and allowed to remain there for 22 hours. Tests were made with the amine added to either distilled water, or to the standard nutrient solution. The readings were made by photoelectric colorimetry of the solutions after suitable dilutions.

The general behaviour of the discs did not agree with that described by Veldstra; dye stuff was given off in appreciable amounts both to water and to nutrient solution even in the controls. Although an increased exosmosis was found in the presence of 6-A.U., indicating an increased permeability, the difference was small and hardly statistically significant.

In order to see the effect of the compound on the vitality of the tissue plasmolysis tests were again performed with both the amine-treated beets and blank samples. In both materials strong plasmolysis was observed, but it ought to be mentioned that in the amine-treated cells concave plasmolysis occurred more often than in the controls.

Conclusions

As it is seen from the results obtained the action of 6-aminoundecane is strongest on the zone of elongation. In this respect it resembles that of growth-regulating compounds, even those of very different structure. This stage seems to be especially sensitive, which in itself is hardly surprising, in view of the delicate changes the cells undergo during the elongation process. The same action as that of 6-A.U. has not been found with other substances, however, not even with the homologous acid, di-n-amylacetic acid. The amine inhibits certainly the root growth, but there was no sign of a specific action on epidermis as with the acid.

The most striking effect of the amine is that on the water conditions. On the basal zone of elongation of the leaves it causes a folding on the chlorenchyma cell walls and a plasmolysis in the colourless parenchyma of the bundle sheath. In this latter case the bundles prevent a contraction of the surrounding parenchyma, and, therefore, with a shortage of the water supply the observed strong plasmolysis will result. This especially localized effect of the substance has been shown not to be caused by an increased transpiration. Furthermore, this would cause a wilting from the margins of the leaves, whereas here only some little exposed parts of the vagina are affected. Therefore, the investigated reaction must depend upon an insufficient water supply through the roots under the influence of the compound used. The decreased water content of the leaves is obviously only the consequence thereof. It is, on the other hand, remarkable that permeability tests have revealed an increased water permeability, at least as regards the exosmosis. Unfortunately, the entrance permeability could not be determined.

Gäumann and Jaag (4) have classified different kinds of wilting in three

groups. 1) Physiologic wilting caused by insufficient supply of water to the roots and characterized by a steady decrease in total water content of the plant. — Wilting caused by infectious diseases is of two types. 2) Toxigenous wilting, caused by chemically active toxins, disturbing the semi-permeability of the cytoplasm and at first enhancing the transpiration by a release of water from the cells. 3) Physically induced wilting, caused by a mechanical clogging of the cell walls, which results in a simultaneous inhibition of water absorption and transpiration. Both types of induced wilting are irreversible. The effect of 6-A.U. is of the same nature as Gäumann's toxigenous wilting but differs in some respects; there is no increase in the transpiration, and the wilting is reversible. It resembles the infectious wilting in its strict localization, and instances have been described of both physical and toxigenous wilting starting in petioles. The differences probably depend upon the fact that 6-A.U. also damages the roots and consequently reduces the water supply. The reversibility may be connected with the low molecular weight of 6-A.U. as compared with the studied natural toxins. It is surprising, however, that no increase in transpiration was observed, because there is obviously a much increased permeability, and this was supposed to be the cause of the increase in the instance of infectious wilting.

Two other possibilities of explaining the impaired water balance ought to be mentioned in this connexion. An unfavourable top:root ratio might give such a result, and the root development is obviously retarded. The same holds true of leaf growth, however, and the change in top:root ratio is not so large as to allow the conclusion that it causes a reduced water supply to the leaves, provided that the resistance to a water transport through the roots is unaltered. Another point to be stressed is that the water flow through the roots may not be only a passive stream but be connected with polar metabolic processes in the roots. Reference is made to the elaborate picture of the water transport through roots presented by Lundegårdh (5). If metabolic activity of some kind is decisive for the water absorption an impaired water balance is possible, apart from the changes of the passive water permeability. In any case, the present observations do not permit any other conclusion than that the amine, directly or indirectly, reduces the water-supplying capacity of the roots.

Nevertheless, the change in the water absorption and the increased permeability together indicate a cytoplasmic action distinguishing the amine from the homologous acids.

Summary

1. The effect of 6-aminoundecane was investigated on various physiologic processes, namely growth, permeability, water balance, and transpiration.

2. During 6 days of observation in 10^{-6} , 10^{-5} , 10^{-4} mol the growth of roots and shoots was gradually inhibited.

3. In 10^{-4} mol concentration, starting from the fourth day on characteristic brownish spots appeared on the surface of the roots involving coagulation of the cytoplasm of the trichoblast cells. At the same stage the leaf bases started wilting locally due to an insufficient water absorption and a reversible loss of turgidity of the elongating cells.

4. The effect of the amine on both parts of the plants does not develop after a certain time of action, but at a fixed age of the seedling.

5. Applying the staining and plasmolytic permeability methods it was found that the amine increases the permeability to water and dyes in roots and shoots. Concave plasmolysis is common in amine-treated materials.

6. There is no significant difference in transpiration between amine-treated leaves and controls. The decrease of the water content of the leaves was quite pronounced, and it was concluded that it depends upon a reduced water supply through the roots. This cannot simply be explained by the change in permeability.

References

1. Burström, H.: Studies on growth and metabolism of roots. I. The action of di-n-amylic acid on root elongation. — *Physiol. Plant.* 2:197. 1949.
2. — Studies on growth and metabolism of roots. II. Di-n-amylic acid and assimilation of nitrate. — *Physiol. Plant.* 2:332. 1949.
3. — Studies on growth and metabolism of roots. III. The adaptation against growth inhibition by aliphatic C_{12} and C_{11} acids. — *Physiol. Plant.* 3:175. 1950.
4. Gäuman, E. und Jaag, O.: Über das toxische und das physikalisch induzierte Welken. — *Phytopathol. Zeitsch.* 16:226. 1950.
5. Lundegårdh, H.: The translocation of salts and water through wheat roots. — *Physiol. Plant.* 3:103. 1950.
6. Veldstra, H.: Actions synergiques des analogues structuraux. — *Bul. Soc. Chim. Biol.* 30:772. 1948.
7. — La balance hydrophile/lipophile des composés organiques, son importance pour leur action physiologique. — *Bul. Soc. Chim. Biol.* 31:1. 1949.
8. Wilske, C. and Burström, H.: The growth-inhibiting action of thiophenoxy acetic acids. — *Physiol. Plant.* 3:58. 1950.

Further Studies on the Utilization of Aspartic Acid, Succinamide, and Asparagine by Green Algae

By

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The utilization of DL-aspartic acid, succinamide, and DL-asparagine as a source of nitrogen for *Scenedesmus obliquus* has been previously studied in detail (Algéus 4). In this paper further reports are given of the investigation which has been extended to cover thirteen additional species. The following changes have been made in the methods previously described (Algéus 1, 4). After inoculation the cultures were placed on a shaking table and shaken during the experiment at a rate of about 120 oscillations per minute. The distance of travel was about 1 cm. As in previous experiments the illumination was continuous, but the light-source consisted of 10 daylight fluorescent tubes placed under the table-top of frosted glass. The cultures were thus illuminated from below. The apparatus will be described in detail later on. The light intensity was approximately 2200 lux, i.e., slightly less than before, but the quantitative composition of the light was different. On account of the above changes the values obtained in the experiment are not comparable with those previously obtained. The number of cells and the nitrogen-content were determined at a time when the cultures were in their maximum stationary phase. The experimental period was about 80 days. Since no determination was carried out of the growth curves of the individual species and as the values obtained do not lie in the logarithmic growth phase, it is impossible to obtain an expression of the growth- and assimilation rates either. The results, however, will be evaluated on the basis of the previous detailed study on *Scenedesmus obliquus*. This organism has also been included in the present experiment.

The growth has been expressed in cells per mm³, with the exception of a few instances where it has been estimated without counting. The signs

+, '++', and so on, in table 1, do probably not require further explanation. The sign \pm in the column headed »Assimilated N» denotes that the amount was too small to be determined with the method used (micro-Kjeldahl). The ammonia in the external medium was as usual determined colorimetrically after distillation at room temperature. \pm in the column headed »Ammonia N» denotes <0.01 mg N per culture.

Table 1 shows that all the species were able to utilize aspartic acid as a source of nitrogen. In a few cases, however, the growth was slight (*Haematococcus*, *Scenedesmus acutiformis*, *Hormidium* sp. 1). Some species, on the other hand, had a high cell count (*Stichococcus*, *Hormidium* sp. 2). *Scenedesmus obliquus*, whose growth and assimilation curve have been previously studied, is of particular interest. The values for this species are almost identical with those obtained in earlier experiments (Alg  us 4). Since it has been established for this species that the final cell count and the nitrogen-content are low on account of slow growth rate and nitrogen-assimilation rate, respectively, there is reason to assume that similar conditions prevail in other species with low final values.

The succinamide can also be utilized by most species. It has previously been demonstrated for *Scenedesmus obliquus* that the rates of growth and nitrogen assimilation are high but that an inhibition occurs at an early stage due to the formation of inhibitory substances during the deamidation. For this reason succinamide gave only slightly higher final values than aspartic acid although the latter is less readily utilized. It appears from table 1 that for *Scenedesmus obliquus* the findings are very much the same under the new conditions of illumination and aeration. The values for the other species can be satisfactorily explained on the assumption that the deamidation rate is high as for *Scenedesmus obliquus* but that the sensitivity to the decomposition products varies. The species can thus be grouped as follows:

1) The prohibitive effect of the inhibitory substances is great. There is only slight growth and nitrogen assimilation, or none whatsoever, in comparison with that caused by aspartic acid: *Chlorella prothothecoides*, *Hormidium* sp. 2, *Zygnema*.

2) The prohibitive effect of the inhibitory substances is less than in group 1. The cell count and the nitrogen-content are approximately as large as with aspartic acid: *Ankistrodesmus*, *Scenedesmus obliquus*, *Scenedesmus dimorphus*, *Scenedesmus quadricauda*, *Stichococcus*.

3) The algae are relatively insensitive to the inhibitory substances. Succinamide is better than aspartic acid: *Haematococcus*, *Scenedesmus acuminatus*, *Scenedesmus acutiformis*, *Hormidium* sp. 1.

4) There is no poisonous effect. The high deamination rate can assert itself fully: *Chlorella vulgaris* B and *Chlorella vulgaris* M.

Naturally, the boundary lines between the groups are not distinct. Although the interpretation submitted in this paper is not the only possible one, it is probably the most likely in view of previous results.

In the earlier study on *Scenedesmus obliquus*, asparagine proved to be a good source of nitrogen. Only the amido-nitrogen was utilized. The assimilation rate was high, eight times greater than with aspartic acid and half as high as with succinamide. Cell division and growth were inhibited only at a fairly late stage. Asparagine gave therefore a comparatively high cell count and a high nitrogen-content per culture. In the experiment represented in table 1, the final values for *Scenedesmus obliquus* are considerably higher than those previously stated, possibly due to the altered conditions of illumination and aeration. *If it is assumed that all the species have largely the same reaction to asparagine as Scenedesmus obliquus in respect to growth rate, nitrogen-assimilation rate, and inhibition, this compound ought to give higher values than aspartic acid. Such is also the case, except for Stichococcus, where the values are the same.*

As is evident from table 1, the nitrogen content of the cells varies fairly greatly both with the source of nitrogen and the species. If the nitrogen-content of the cells is compared with that in the glycocoll and alanine cultures (Algéus 2, 3), the general conclusion is that those species which have previously been shown to be rich in nitrogen have a high nitrogen-content also in the experiments presented here, provided that utilizable nitrogen is available. Excess ammonia in the medium can be considered an indicator of this condition.

The algae could be divided into two groups according to their relation to glycocoll and DL-alanine, called the *Scenedesmus* and *Chlorella* types. In the former group the capacity for deamination was great and excess nitrogen could be recovered in the culture medium in the form of ammonia. In several cases the ammonia content amounted to 1 mg, or more, per culture. In the latter group, the nitrogen assimilation was limited by the deamination and the solutions were free from ammonia. All the species react in practically the same way to aspartic acid. The solutions are free from ammonia, with some few exceptions. There is reason to believe that nitrogen is the limiting factor. *Thus, in their relationship to aspartic acid the species can be said to belong to the Chlorella type.* It should be observed, however, that the absence of ammonia need not be due to a restricted deamination process. The aspartic acid can also be utilized through a transamination of the following type (Cohen 5, Leonard and Burris 6), where no free ammonia occurs:

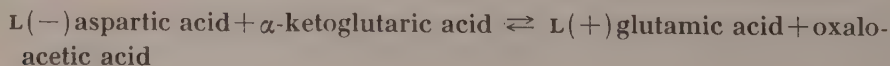


Table 1. *Growth, nitrogen metabolism, and pH in cultures with aspartic acid, succinamide and asparagine.*

Species	Aspartic acid					Succinamide					Asparagine				
	Growth, cells per mm ³	Assimilated N, mg per culture	Ammonia-N, mg per culture	N-content per cell, mg · 10 ⁻¹⁰	Final pH	Growth, cells per mm ³	Assimilated N, mg per culture	Ammonia-N, mg per culture	N-content per cell, mg · 10 ⁻¹⁰	Final pH	Growth, cells per mm ³	Assimilated N, mg per culture	Ammonia-N, mg per culture	N-content per cell, mg · 10 ⁻¹⁰	Final pH
<i>Haematococcus pluvialis</i>	+	±	1.19	—	7.4	+	0.24	0.04	—	7.5	+	0.25	0.91	—	6.9
<i>Chlorella vulgaris</i> B	+	0.01	0.18	—	7.2	+	0.22	0.03	—	7.6	+	0.43	0.09	—	7.0
<i>Chlorella vulgaris</i> M	2,400	0.02	0.02	2.	7.1	21,000	0.13	0	1.2	7.0	3,500	0.03	0	2.	6.9
	1,500	0.03	0	4.	7.0	19,000	0.14	0	1.5	7.0	3,900	0.05	0	3.	6.7
	1,100	0.03	0	6.	6.9	13,000	0.08	0.03	1.	6.5	19,000	0.27	0	2.8	7.0
	1,300	0.15	0	23.	6.8	14,000	0.10	0.04	1.4	6.5					
<i>Chlorella protothecoides</i>	5,700	0.10	0	3.5	6.9	2,300	0.04	0.28	4.	6.7	12,000	0.73	±	12.	5.1
	4,700	0.06	0	3.	6.8	1,800	±	0.41	—	6.9					
<i>Ankistrodesmus falcatus</i>	6,300	0.82	0	26.	7.2	6,000	0.27	0.05	9.0	7.3	6,300	2.03	0.53	64.4	9.0
	6,300	0.75	0	24.	7.4	4,900	0.23	0.02	9.4	7.2	5,700	2.05	0.45	71.9	8.9
<i>Scenedesmus obliquus</i> ...	5,800	0.21	0	7.2	7.6	7,200	0.28	0	7.8	7.2	36,000	3.35	0.03	18.6	7.1
	5,900	0.19	0	6.4	7.6	14,000	0.49	0	7.0	7.5	39,000	3.31	0	17.0	7.1
<i>Scenedesmus dimorphus</i>	6,700	0.28	0	8.4	7.7	6,000	—	—	—	6.9	42,000	3.81	1.26	18.1	8.6
	8,400	0.37	0	8.8	8.2	7,700	—	0.05	—	6.9	59,000	1.72	1.43	5.83	8.5
<i>Scenedesmus acuminatus</i>	1,300	0.14	0	21.	7.1	3,300	0.40	0.03	24.	7.3	2,400	2.74	0.66	228.	7.9
	1,500	0.15	0	20.	7.4	4,300	0.84	0.03	39.	7.5	2,100	2.83	0.63	270.	7.9
<i>Scenedesmus acutiformis</i>	250	0.13	0	100.	7.4	600	0.14	0.18	47.	7.0	400	2.84	±	1420.	5.2
	300	0.11	±	73.	7.2	500	0.13	0.22	52.	7.0	500	2.73	±	1090.	5.2
<i>Scenedesmus quadricauda</i>	3,700	0.34	0	18.	7.5	5,000	1.15	0	46.0	7.4	17,000	5.62	0	66.1	6.9
	5,300	0.29	0	11.	7.6	4,700	0.88	0	37.	7.4	15,000	—	0	—	6.9
<i>Stichococcus bacillaris</i> ...	37,000	0.30	0	1.6	7.2	31,000	0.27	0.04	1.7	6.9	38,000	0.43	0	2.3	4.4
	31,000	0.28	0	1.8	7.4						34,000	0.38	0	2.2	4.4
<i>Horridium</i> sp. 1	±	0.06	0	—	7.2	+	0.08	0.83	—	8.3	+	0.11	0	—	6.4
	±	0.03	0	—	7.2	+	0.08	0.01	—	7.1	+	0.08	0	—	6.8
<i>Horridium</i> sp. 2	49,000	0.95	0.15	3.9	7.5	9,600	0.13	0.01	2.7	6.2	31,000	1.96	0.86	12.6	6.9
	38,000	1.23	±	6.47	8.6	7,900	0.08	0.02	2.	6.2	34,000	1.89	0.46	11.1	6.9
<i>Zygnema</i> sp.	+	0.27	0	—	7.6	0	0	±	—	7.1	+	0.31	0	—	4.9
	+	0.15	0	—	7.7	0	0	0	—	7.1	+	0.39	0	—	5.1

There are many indications that aspartic acid does not occupy the central position in the nitrogen metabolism of plants as has often been previously assumed, but that this role is filled by glutamic acid. If a reaction of the abovementioned type limits the assimilation of nitrogen when aspartic acid is used as the source of nitrogen, experiments with glutamic acid must yield higher values for the rates of assimilation and cell division. Investigations are in progress.

Finally, it should be observed that ammonia occurs in usually small but varying quantities when succinamide and asparagine are used as the source of nitrogen. In these cases, however, the amido-nitrogen of the organic compound is split off, as has been previously demonstrated for *Scenedesmus obliquus* (Algéus 4). The mechanism of deamidation must, however, be quite separate from the processes of deamination and transamination discussed in the previous paragraphs. In this connexion it is noteworthy that cultures with asparagine and succinamide normally showed a green to dark green colour (the *Chlorella* species, however, were pale green), while the cultures with aspartic acid at the end of the experiment were practically always pale green, some even white. The cells were still viable, however, and after being transferred to an inorganic culture medium they grew with a normal appearance.

Summary

1. Fourteen species of green algae representing the genera of *Haematococcus*, *Chlorella*, *Ankistrodesmus*, *Scenedesmus*, *Stichococcus*, *Hormidium*, and *Zygnema* have been studied with regard to their relationship to aspartic acid, succinamide, and asparagine in pure cultures. The results are discussed in connexion with the author's earlier detailed study on *Scenedesmus obliquus*.

2. All the species were able to utilize aspartic acid. In the majority of cases growth and the amount of assimilated nitrogen were moderate; in some cases, however, they were slight. Judging from previous experiments this fact is assumed to be due to a limited capacity of the algae for transaminating or deaminating the amino acid.

3. The species could also utilize succinamide. The variation of the values from one species to another is best explained by an assumption of a high deamidation rate but varying sensitivity to the inhibitory effect of the decomposition products.

4. Asparagine is throughout a better source of nitrogen than aspartic acid, which, as in *Scenedesmus obliquus*, may be due to a high rate of deamidation and a slight poisonous effect of the decomposition products.

References

1. Algéus, S.: Glycocoll as a Source of Nitrogen for *Scenedesmus obliquus*. — *Physiol. Plant.* 1: 65. 1948.
2. — The Deamination of Glycocoll by Green Algae. — *Physiol. Plant.* 1: 382. 1948.
3. — Alanine as a Source of Nitrogen for Green Algae. — *Physiol. Plant.* 2: 266. 1949.
4. — The Utilization of Aspartic Acid, Succinamide and Asparagine by *Scenedesmus obliquus*. — *Physiol. Plant.* 3: 225. 1950.
5. Cohen, P. P.: Transamination with Purified Enzyme Preparations (Transaminases). — *J. Biol. Chem.* 136: 565. 1940.
6. Leonard, M. J. K. and R. H. Burris: A Survey of Transaminases in Plants. — *J. Biol. Chem.* 170: 701. 1947.

On the Potassium Management of the Yeast Cell in the Presence of Weak Acids

By

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Earlier investigations showed that potassium is removed from the yeast cell when NaF beyond a certain concentration is added to a yeast suspension (Malm 1940, 1945, 1947). It was further established that hydrogen fluoride thereby enters the yeast cell. Also, erythrocytes liberate potassium after the addition of NaF to an erythrocyte suspension, as was shown by Wilbrandt (1937) and Davson (1941).

To explain this potassium emergence, Davson and Wilbrandt assumed that NaF in an indirect way causes an increase in the potassium permeability of the erythrocyte membrane. They assumed that the influence of NaF on the normal course of glycolysis caused an accumulation of an intermediary metabolite or some other normally non-occurring substance which is lipoid soluble and surface active. This causes a change in the structure of the erythrocyte membrane which leads to an increased potassium permeability.

The author's investigations, however, do not argue in favor of a permeability change as the cause of the potassium emergence. They indicate rather that the potassium emergence is the consequence of a decreased pH in the cell. If it were due to a change in permeability, one would expect that when the permeability for potassium is strongly increased the permeability for sodium would also increase. The yeast cell normally contains but traces of sodium and is very little permeable to this ion. The cause for this lies in the ionic selectivity of the cell, which, as in cells in general, probably is connected with the structural peculiarities of the protoplasm. It is chiefly the size of the ion which determines which kind of ions in the surrounding medium will win the competition for the non-diffusible ions with the opposite charge in the cell. The diameter of the hydrated potassium ion is 7.6 Å, and

that of the hydrated sodium ion 11.2 Å. In the presence of NaF, when the potassium permeability apparently is greatly increased, one would also expect an increased diffusion of sodium ions into the cell from a medium containing sodium. This is, however, not the case (see Malm 1947).

Experiments with radioactive potassium, K^{42} , tend to indicate that under normal conditions erythrocytes (Brooks 1938, Hevesy 1939) as well as yeast cells (Hevesy and Nielsen 1941) — even without exogen supply of nutrition — are permeable to K. A permeability hindrance seems therefore not to be present. This can only be demonstrated with labelled potassium. Judging from chemical analysis only, the above-mentioned cells would appear to be impermeable to potassium ions because the resting cells always maintain a non-variable amount of potassium.

Chemically noticeable changes of the potassium content of cells or tissues are mainly observed in following cases. An increased power to bind K takes place with a rich supply of nutrition and, above all, with growth. From a clinical point of view, the strong power of tumor cells to accumulate potassium is of great interest. A decreased power to bind potassium is a well known symptom of diabetes. A prolonged excitation of the widely specialized kinds of cells, such as muscles and nerves, can result in a potassium emergence. A potassium emergence from the cell occurs by cytolysis, in which a general salt leakage takes place. A comparatively simple method to cause a potassium emergence is to add NaF to a cell suspension, as already mentioned in the introduction. It is not a question of cytolysis in this case since as soon as the fluoride is washed out, the metabolism again behaves more or less normal (Malm 1947).

Since by the treatment of the yeast cell with NaF the pH of the surrounding medium in our experiments always was more or less lower than the pH of the cell, there occurred by the entrance of HF an accumulation of hydrogen and fluoride ions in the cell (Malm 1947). The potassium emergence consequently could be caused directly or indirectly by one of the three components HF, H^+ , or F^- .

For a choice between these three components, the following is to be noted. By the well-known phenomena of inhibition of respiration and fermentation of different cells in the presence of NaF, the attention of the different research workers has been directed practically only on the fluoride ion (see Malm 1947, p. 177). In an earlier paper (Malm 1947), however, calculations based upon chemical fluoride analyses indicate that the pH of the cell seems to decrease by the permeation of HF. The pH decreases progressively as the concentration of the added NaF is increased. It is to be noticed that the inhibition phenomena occurs at a NaF concentration at which both an obvious potassium emergence and a pH decrease of about one unit takes

place. There is still another circumstance to be noticed, that is, the simultaneous break down of carbohydrates (Malm 1947, p. 160). Further investigations on this topic will be presented in a coming paper.

It is evident that the apparant large changes in the pH in the cell would not leave the enzyme system of the cell unaffected. Enzymes with an optimum activity at the normally prevalent pH in the cell would thus more or less decrease in their action. For other enzymes, however, the diminished pH might cause a shift toward an activity optimum. In this connection the observation of Suomalainen (1948) should be considered, that is, the α -glucosidase from plasmolysates of baker's yeast has two optima, one at pH 7 and one at pH 4.

Thus a carbohydrate breakdown could be expected in advance regardless of whether or not some of the enzymes are specifically affected by HF or F by the formation of complexes or otherwise. A carbohydrate breakdown in its turn could also be expected to influence the potassium management, since the latter seems to be closely connected with the carbohydrate metabolism. The potassium excretion could therefore be caused by the hydrogen ions which appear in the cell by the dissociation of HF.

The potassium of the resting yeast cell is, under normal conditions, very strongly fixed in the cell. According to our experience, for instance, a washing of the yeast cells for several hours with distilled water causes practically no potassium excretion. The total potassium, however, must be present in the cell in the form of ions. This is shown by the investigations of Conway et al. (Conway and Boyle 1939; Boyle and Conway 1941) who was able to demonstrate that the total potassium of the yeast cell can be exchanged for ammonium ions. The total cell potassium is also exchangeable with K^{42} in the surrounding medium (Hevesy and Nielsen 1941; Malm 1945). The reason that potassium does not escape from the cell under normal conditions is perhaps to be found in the assumption that potassium is electrostatically attracted to non-diffusible compounds. When these non-diffusible compounds are for some reason transformed into more or less diffusible ones, one would expect potassium diffusion from the cell.

In this connection a few lines from a paper by Conway (1947, p. 25) will be quoted here: »With any cellular breakdown of non-diffusible anions to diffusible, potassium will be released, and absorbed whenever they are synthesized. We may thus speak of 'active' absorption of potassium into the cell, and 'active' excretion of potassium therefrom in various metabolic phases. It is also to be noted that acidification or alkalinization of the cellular contents will be a potent cause of K exchanges. But in all such 'active' exchanges, the K ions will have only passively accompanied the changing electrostatic pattern.»

Also from osmotic considerations it seems likely that a large part of the potassium in the yeast cell is electrostatically attracted to non-diffusible compounds. In an earlier paper (Malm 1947, p. 161) it was demonstrated, on the base of the total potassium and phosphorus amounts of the yeast cell, that the total potassium could practically completely be present as primary orthophosphate, i.e., in the most probable form by the pH prevalent in the cell (about 6). As is well known the phosphate in the cell is present mainly in esters. The non-diffusible compounds considered in this connection would thus be acid phosphate esters. The fresh intact yeast cell is practically impermeable towards the known phosphate esters, isolated from the cell. From the above quote one could assume that the activity of the potassium ions of the cell must be considerably depressed. The osmotic behaviour verifies this assumption. The yeast cell is isotonic with an approximately 0.1 M NaCl-solution (Malm 1947). The potassium concentration, when calculated from the total water content of the yeast cell, is 0.29 M, i.e., about 3 times as strong a concentration as the isotonic NaCl-solution. In addition, other positively charged ions than potassium occur in the cell, for instance, Ca^{++} and Mg^{++} , even though K^+ quantitatively predominates.

The hydrogen ions introduced into the cell could thus cause a potassium emergence by means of the effect of a change in the pH on the enzyme activity by which non-diffusible compounds are transformed into diffusible. van Laer (1920) suggested that the capacity of enzymes to hydrolyse compounds is only a modification of the ordinary influence of hydrogen ions. An accelerated activity in the presence of enzymes is to be ascribed to the high concentration of hydrogen ions which are adsorbed on the surface of these enzymes. The hydrogen-ion concentration of the enzyme surface is in equilibrium with that of the surrounding medium. Therefore, the maximum activity of the enzyme is reflected in the pH of the medium. This view agrees with the investigations of Danielli (1937, 1944) concerning the concentration of ions in the immediate vicinity of a charged surface, whereby it was demonstrated that the hydrogen-ion concentration at a surface may be up to 10 or even 100 times greater than in the surrounding medium bathing this surface. These circumstances have already been dealt with in an earlier paper (Malm 1947, p. 130 and 179).

It could also be considered that the potassium emergence in the presence of HF is directly caused by the hydrogen ions formed by the dissociation of HF in the cell. That is, an exchange between hydrogen ions and potassium ions of the cell could take place. Such an exchange, but in the opposite direction, i.e., potassium ions in the medium exchanging with hydrogen ions from the cell, has been demonstrated by Conway and O'Malley (1944; 1944 a) as well as by Rothstein and Haeger (1946).

Thus, if the hydrogen-ions, which appear in the cell by the penetration of HF, directly or indirectly cause the potassium emergence, it should be expected that other weak acids would also be able to cause a potassium loss. However, acids which permeate into the cell and which are not attacked by the enzymes of the cell can cause this K emergence. It is important that the acid be able to penetrate into the cell because a decrease of the pH in the surrounding medium alone, caused by an acid for which the cell is impermeable, does not change the pH of the cell.

The aim of the present experiments was thus to examine the influence of different weak acids on the potassium content of the yeast cell.

Methods

Baker's yeast, cultivated under strong aeration, was used in the present experiments. This yeast, derived from the Swedish Yeast Company at Rotebro, Stockholm, is characterized as 0 5 in the author's earlier papers (e.g. Malm 1947).

The yeast suspensions were always prepared in a ratio of 3 g. yeast in 10 ml. solution. When not otherwise mentioned, the suspensions were shaken in Fernbach flasks — in order to have a large surface to assure aerobe conditions — in a water thermostat at 25° C. The yeast samples were removed from the suspension after certain times and were immediately filtered from the suspension through a glass filter (G4 Schott). The yeast samples were then washed on the filter three times and dried by 100° C. The dry yeast was pulverized and certain amounts were weighed out for a potassium analysis.

The potassium determinations were performed partly with the aid of measurements of the activity of yeast marked with K^{42} , and partly in the chemical way (Nydahl 1932). K^{42} was added as $K^{42}Cl$ to the yeast suspension and the marking performed as already mentioned in earlier papers (Malm 1945, 1947).

The K^{42} placed at the author's disposal was prepared partly in the cyclotron of Nobelinstitutet för Fysik, Stockholm, and partly in the cyclotron of Institutet för Teoretisk Fysik, Copenhagen. The author wishes to take the opportunity here of expressing her appreciation for these samples.

Since a Beckman flame spectrophotometer has become available in the institute, part of the potassium determinations were performed by this method. Also, some of the earlier results obtained with the radioactive and chemical methods were repeated with this method.

The pH-determinations of the filtered solutions were performed with a glass electrode. The apparatus used was a valve potentiometer (Radiometer, Copenhagen).

The potassium-binding capacity of the yeast cell in the presence of a number of weak acids

The following weak acids have been examined with regard to their influence on the potassium-binding power of the yeast cell: formic, acetic, propionic, butyric, isobutyric, lactic, β -hydroxy-butyric, succinic, tartaric, and citric acid, in addition to the already earlier examined hydrogen fluoride.

The solutions of the weak acids or their sodium salts were, when necessary, adjusted with the aid of NaOH or HCl to the desired pH. Generally the experiments were carried out with a pH of between 7 and 3 in the medium. No special buffering of the medium was employed as the buffering capacity of the weak acids and their sodium salts was sufficient for the present purpose, and it was of interest to have the acid concerned or the mixture of the acid and its sodium salt as the only compounds present in the suspension medium. It was, moreover, of interest to keep an insufficient buffering in the medium since then only a measurement of the pH of the filtered suspension solution was necessary to show if potassium had escaped from the cell or not.

Generally 0.1 M solutions were used. Especially at low pH, where the dissociation of the acids is more or less repressed, the solution surrounding the cells thus became slightly hypotonic in relation to the cell (cf. page 379).

A suspension of yeast in 0.1 M NaCl generally served as a control for the experiments. Since earlier investigations (Malm 1947) showed the potassium content of the yeast cell not to be influenced by different pH in the medium alone, the control generally was carried out only at one pH, by neutral reaction. Furthermore, since earlier investigations have shown that practically no potassium escapes even if the yeast cells are shaken for several hours in a 0.1 M NaCl solution, samples of the control were not taken as frequently as of the suspensions to be examined. Since the solution of the control was unbuffered, the pH-readings of this solution could not be carried out with the same accuracy as in the case of the other solutions. Therefore, generally, only the first decimal of the pH value of the control solution is taken up in the tables.

Formic acid

The yeast was suspended in 0.1 M formic acid solutions of 5 different pH values. Samples were removed immediately and after the lapse of 1 and 4 hours. A suspension of yeast in 0.1 M NaCl served as a control.

Table 1 shows the results of a typical experiment. From this it appears that potassium escapes from the cell in the presence of formic acid when the pH of the solution from the beginning is 3.6 or less. At the same time, the pH of the solution increases.

The formic acid can, when sufficiently diluted, be attacked by the yeast cell. According to Ducleaux the yeast cell consumes 0.04—0.07 % formic acid (Ducleaux 1892; see also Franzen 1912). In the present experiments — as mentioned above — 0.1 M formic acid was used. It was established, with the aid of Warburg's micro-manometric method, that formic acid of this concentration is not attacked by the yeast.

Table 1. *Effect of 0.1 M formic acid on the potassium content of the yeast cell.*

No.	Time of shaking the yeast cells in formic acid					
	0 min.		60 min.		240 min.	
	mg.K/100mg. dried yeast	pH of the solution	mg.K/100mg. dried yeast	pH of the solution	mg.K/100mg. dried yeast	pH of the solution
control	2.18	7.0	2.17	—	2.15	6.28
1		5.20	2.27	6.40	2.14	7.98
2		4.54	2.30	5.40	2.22	6.73
3		4.09	2.31	4.68	2.31	4.52
4		3.61	1.86	3.98	1.47	4.19
5		3.17	1.03	3.77	0.52	3.91

Table 2. *Rate of permeation of 0.1 M formic acid into the yeast cell.*

Time in min.	% formic acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
1	35.0	2.7	2.52
4	42.1	4.3	2.61
8	40.5	6.4	2.69
12	43.8	8.4	2.79
20	41.2	12.1	2.94

According to investigations by Wieringa (1930), formic acid easily penetrates the yeast cell. His experiments were carried out with 0.1 M formic acid. There is, however, no quantitative information about the rate of permeation or about the methods used. The author's own attempts to analyze the formic acid-formate solutions in which yeast has been shaken have hitherto met with difficulties. Therefore, until now the author has only been able to determine the permeation rate of the formic acid in pure 0.1 M formic acid solutions, without sodium formate, where the actual amount of acid can be simply found by titration with NaOH. The pH of a 0.1 M formic acid solution is 2.3. It appeared that an equilibrium in the distribution of formic acid between medium/cell was attained during the first minutes.

Table 2 shows the amount of formic acid that disappeared after various times expressed in per cent of the original concentration of the solution after due correction for the dilution caused by the intercellular water of the yeast cells. The third column of Table 2 shows the amount of potassium that escaped in per cent of the total potassium of the cell. In the present case, the escaped potassium is determined directly in the filtered solution with the aid of the flame-spectrophotometer.

Further results on the potassium escape in the presence of formic acid, as well as more detailed circumstances, will be published in a following paper.

Table 3. *Effect of 0.1 M acetic acid on the potassium content of the yeast cell.*

No.	Time of shaking the yeast cells in acetic acid					
	0 min.		60 min.		240 min.	
	mg.K/100mg. dried yeast	pH of the solution	mg.K/100mg. dried yeast	pH of the solution	mg.K/100mg. dried yeast	pH of the solution
control	2.18	7.0	2.19	6.96	2.07	7.25
1		5.0	2.23	5.01	2.17	5.15
2		4.4	2.23	4.25	2.35	4.26
3		3.8	2.30	3.67	2.27	3.65
4		3.2	2.10	3.38	2.24	3.44

Table 4. *Rate of permeation of 0.1 M acetic acid into the yeast cell.*

Time in min.	% acetic acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
1	22.1	0.3	3.08
4	23.4	0.5	3.13
8	24.4	0.7	3.14
12	25.3	0.9	3.16
20	27.0	1.6	3.18

Acetic acid

The yeast was suspended in 0.1 M acetic acid solutions of 4 different pH values. After 1 and 3 hours samples were removed. A suspension of yeast in 0.1 M NaCl again served as control. The potassium content was analysed by the chemical method.

As can be seen from Table 3, the acetic acid at the pH used does not exert any influence upon the potassium-binding power of the yeast cell. Also, the pH-values of the solutions are on the whole unchanged. Nevertheless, the increase of the pH by 0.22 pH-units after 3 hours, in the case of the solution with the lowest pH, indicates that acid has disappeared from the solution.

Under certain circumstances the yeast cell is able to turn over acetic acid. In the present case, however, the pH-determinations indicate that no turnover worth mentioning has occurred. At a rapid turnover the pH-values would have increased, because in all cases the solutions were made up by a mixture of acetic acid-sodium acetate, and the sodium ion is not permeable.

According to Wieringa (1930), acetic acid permeates less easy than formic acid into the yeast cell. No quantitative information is, however, given. Also in this case, therefore, experiments were carried out in which the yeast

was shaken in a pure 0.1 M acetic acid solution ($\text{pH}=2.84$) and the amount of the acid in the solution after various times determined by titration with NaOH. The results of such experiments are found in Table 4. The potassium is, again — as in the analogous experiments with formic acid — analysed in the filtered solution with the aid of the flame-spectrophotometer.

Here also, as in the case of the formic acid, a distribution equilibrium for acetic acid between solution/cell is rapidly established. The amount permeated acid at equilibrium is, however, considerably smaller in the latter case.

Propionic acid

Yeast shaken in 0.1 M propionic acid showed, even after 4 hours treatment, practically no potassium loss. The experiments were carried out in a pH-range of about 5 to 3. The control was prepared in the same way as in the preceding experiments. The results are recorded in Table 5. The analyses were carried out by the chemical method.

As can be seen from Table 5, practically no potassium has escaped from the cell. Also, the pH-values of the solutions have not changed noticeable during the experiment.

The experiment was repeated with 0.2 M propionic acid. The results are recorded in Table 6.

As can be seen from Table 6 in this case also no potassium has escaped. An exception occurs in No. 6, which, however, must be ascribed to pure chance, since otherwise a potassium loss should also have occurred in No. 7 and 8, where a still lower pH was used. Also, the pH-values of the solutions are unchanged during the experiment, with the exception of the one with the lowest pH.

Judging from the comparatively high lipod solubility of the propionic acid (see p. 394), one would expect the acid to penetrate the cell readily. According to a personal communication by professor S. J. Ørskov, propionic acid does in reality permeate into the yeast cell and has even been found unchanged in the cell. It appeared, however, that Ørskov in his experiments had used a hydrogen-ion concentration of pH 2. Therefore, the author's experiments were repeated at this pH.

Again a 0.1 M propionic acid solution was used. Since the pure propionic acid at this concentration has a pH of 2.88, the pH was adjusted to pH 2 with the aid of HCl. The control was prepared in the same manner as in the preceding experiments. Table 7 shows a typical result. The potassium analyses were carried out by the radioactive method.

It appears from Table 7 that the yeast cells lose potassium by shaking in a propionic acid solution at pH 2, although only to a small extent.

Table 5. *Effect of 0.1 M propionic acid on the potassium content of the yeast cell.*

No.	Time in hours for treatment of the yeast cell with propionic acid	mg.K/100mg. dried yeast	pH of the solution
control	—	2.50	—
—	—	—	4.74
1	1	2.46	4.87
2	4	2.46	4.95
—	—	—	4.07
3	1	2.60	4.09
4	4	2.64	4.02
—	—	—	3.52
5	1	2.59	3.47
6	4	2.59	3.39
—	—	—	2.93
7	1	2.43	3.10
8	4	2.53	3.01

Table 6. *Effect of 0.2 M propionic acid on the potassium content of the yeast cell.*

No.	Time in hours for treatment of the yeast cell with propionic acid	mg.K/100mg. dried yeast	pH of the solution
control	—	2.55	—
—	—	—	4.74
1	1	2.68	4.83
2	4	2.58	4.76
—	—	—	4.07
3	1	2.70	4.14
4	4	2.60	4.08
—	—	—	3.51
5	1	2.48	3.64
6	4	2.07	3.58
—	—	—	2.78
7	1	2.56	3.14
8	4	2.56	3.19

Table 7. *Effect of 0.1 M propionic acid on the potassium content of the yeast cell at a pH of about 2.*

No.	Time in min.	impulses/min. per 100mg dried yeast	K, in % of total cellular K, that escaped from the cell	pH of the solution
—	—	—	—	1.92
Control	180	860	0	—
1	2	851	1.0	2.14
2	30	878	0	2.19
3	60	848	1.4	2.21
4	120	833	3.2	2.26
5	180	784	8.9	2.35

Table 8. *Rate of permeation of 0.1 M propionic acid into the yeast cell.*

Time in min.	% propionic acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
—	—	—	2.88
1	25.4	0.8	3.15
4	25.4	0.9	3.07
8	24.7	1.1	3.15
12	27.0	1.3	3.09
20	27.6	1.4	3.09

Since no quantitative information on the permeation-ability of the propionic acid into the yeast cell could be found in the literature, we again carried out an experiment whereby the yeast was shaken in a pure 0.1 M propionic acid solution (pH=2.88) and the acid concentration of the solution at various times was determined by titration with NaOH. Escaped potassium was again analysed in the solution with the aid of the flame-spectrophotometer. The results are recorded in Table 8.

Table 8 shows that a distribution equilibrium for the acid between medium/cell rapidly is established. The percentage penetrated acid at equilibrium is the same as in the case of acetic acid.

Butyric acid

Yeast, shaken up to 4 hours in a 0.1 M butyric acid solution, showed no more potassium loss than in the case of propionic acid. The results are recorded in Table 9. The control was prepared in the same way as in the preceding experiments. The analyses were carried out by the chemical method.

Table 9 shows that practically no potassium has escaped in the presence of 0.1 M butyric acid.

The experiment was repeated with a 0.2 M butyric acid solution. The analysis were carried out by the radioactive method.

As can be seen from Table 10, a noticeable potassium loss has taken place in the samples with lower pH, when the butyric acid concentration was 0.2 M. In this case the pH of the solution also increased.

Similiar to the case for the propionic acid, one would expect the butyric acid to penetrate easily into the cell owing to its high lipid solubility (see p. 394). According to Ørskov butyric acid also easily penetrates into the yeast cell. However, in this case also Ørskov used solutions with a hydrogen-ion concentration of pH 2, and therefore the experiments above were repeated at this pH. A 0.1 M solution was used. The control was prepared in the

Table 9. *Effect of 0.1 M butyric acid on the potassium content of the yeast cell.*

No.	Time in hours for treatment of the yeast cell with butyric acid	mg.K/100mg. dried yeast	pH of the solution
Control	—	2.10	—
1	1	2.11	4.70
2	4	2.10	4.82
3	1	2.35	4.80
4	4	2.14	4.04
5	1	2.10	4.11
6	4	2.03	4.04
7	1	1.92	3.47
8	4	2.06	3.59
			2.88
			3.23
			3.18

Table 10. *Effect of 0.2 M butyric acid on the potassium content of the yeast cell.*

No.	Time in hours for treatment of the yeast cell with butyric acid	K, in % of the total cellular K, that escaped from the cell	pH of the solution
Control	4	0	—
1	1	0	4.70
2	4	0	4.81
3	1	0	4.77
4	4	5.5	3.98
5	1	4.5	4.15
6	4	0	4.15
7	1	13.2	3.39
8	4	13.1	3.65
			3.70
			2.75
			3.27
			3.46

usual manner. Table 11 shows a typical result. The potassium analyses were carried out by the radioactive method.

As can be seen from Table 11, even after 3 hours only very little potassium has escaped from the cell. The pH of the solution, however, has increased by 0.4 unit. This could be explained by a decrease in the butyric acid in the medium.

Once again no quantitative figures could be found in the literature so an experiment was carried out whereby the yeast cells were shaken in a pure 0.1 M butyric acid and the acid concentration of the solution was titrimetrically determined at different times. The results of such an experiment are recorded in Table 12. The figures in the table are corrected with regard

Table 11. *Effect of 0.1 M butyric acid on the potassium content of the yeast cell at a pH of about 2.*

No.	Time in min.	Impulses/min. per 100mg.	K, in % of the total cellular K, that escaped from the cell	pH of the solution
—	—	—	—	1.93
Control	180	866.2	0	—
1	2	878.3	0	2.01
2	30	836.3	4.7	2.17
3	60	878.8	0	2.19
4	120	858.2	2.4	2.24
5	180	850.1	3.2	2.30

Table 12. *Rate of permeation of 0.1 M butyric acid into the yeast cell.*

Time in min.	% butyric acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
—	—	—	2.85
1	24.4	2.4	3.18
4	25.8	2.8	3.19
8	25.0	3.1	3.27
12	26.1	3.2	3.28
20	27.4	3.6	3.20

to the dilution of the solution by the intercellular water of the yeast cells, as was the case for the preceding acids.

From Table 12 it appears that even in this case a distribution equilibrium of the acid between medium/cell is rapidly reached. The percentage permeated acid at equilibrium is the same as in the case of acetic and propionic acid.

Experiments with the micro-manometric method of Warburg showed that butyric acid was not turned over by the yeast cells under the experimental conditions used.

Isobutyric acid

When yeast was shaken in isobutyric acid the same general results were obtained as when butyric acid was used. Here again the permeation rate of the acid was titrimetrically established in a pure 0.1 M isobutyric acid solution. The results are recorded in Table 13. The potassium loss was again determined by analysis of the solution with the aid of the flame-spectrophotometer.

Table 13 shows that the establishment of an distribution equilibrium for isobutyric acid between solution/cells is reached as fast as in the cases of acetic, propionic and butyric acids. Also the percentage permeated acid is the same as for the above mentioned acids.

Table 13. *Rate of permeation of 0.1 M isobutyric acid into the yeast cell.*

Time in min.	% isobutyric acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
—	—	—	2.85
1	24.4	2.4	3.18
4	25.8	2.8	3.19
8	25.0	3.1	3.27
12	26.1	3.2	3.28
20	27.4	3.6	3.20

Lactic acid

The yeast was shaken in 0.1 M lactic acid solutions at 5 different pH-values. Samples were taken after 1 and 3 hours. A suspension of yeast in a neutral 0.1 M NaCl solution served as control. The analyses were carried out by the chemical method. The results of such an experiment are shown in Table 14.

As can be seen in Table 14, the lactic acid does not cause a potassium loss from the cell. The yeast cell easily turns over lactic acid, and therefore the acid has disappeared more or less completely from the solution after 3 hours. Since the solutions at the pH used were composed of a mixture of sodium lactate-lactic acid, and since the sodium ion does not permeate, the pH-values of the solutions are strongly increased.

Table 14. *Effect of 0.1 M lactic acid on the potassium content of the yeast cell.*

No.	Time for shaking the yeast cells in lactic acid					
	0 min.		60 min.		240 min.	
	mg.K/100mg. dried yeast	pH of the solution	mg.K/100mg. dried yeast	pH of the solution	mg.K/100mg. dried yeast	pH of the solution
Control	2.04	7.00	2.11	—	2.04	6.85
1		5.07	2.10	6.93	2.07	6.75
2		4.27	2.10	4.68	2.18	7.86
3		3.65	2.16	3.83	2.08	7.49
4		3.32	2.34	3.47	2.12	7.04
5		2.98	2.04	3.13	2.20	4.49

 β -hydroxy-butyric acid

From the clinical point of view, the influence of the weak acid β -hydroxy-butyric acid upon the potassium management of the cell is of interest because it is assumed that in diabetes, β -hydroxy-butyric acid, along with

Table 15. *Rate of permeation of 0.0964 M β -hydroxy-butyric acid into the yeast cell.*

Time in min.	% β -hydroxy-butyric acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
—	—	—	2.85
1	24.4	2.4	3.18
4	25.8	2.8	3.19
8	25.0	3.1	3.27
12	26.1	3.2	3.28
20	27.4	3.6	3.20

other acids, causes a potassium loss from the cells by which the acid becomes neutralized.

The yeast was shaken in a pure 0.0964 M β -hydroxy-butyric acid (pH=2.52). The concentration of the acid at different times was obtained by titration with NaOH. The potassium content of the solution was analysed with the flame-spectrophotometer. The results of such an experiment are recorded in Table 15.

From Table 15 it can be seen that the establishment of a distribution equilibrium for the acid between the surrounding medium/cell is not reached as fast as in the case of, for instance, butyric acid. The degree of the potassium loss of the cell, however, is about the same.

Succinic acid

As already demonstrated in an earlier paper (Malm 1947), the treatment even for several hours of yeast cells with succinic acid-sodium succinate mixtures of different pH does not cause any potassium escape. In the paper mentioned, it was also discussed, in detail, whether or not the succinic acid is able to permeate into the yeast cell. Under the experimental conditions used (yeast conc. of the suspension=23 %, pH=3 to 7, succinic acid-sodium succinate conc.=0.1 to 0.2 M) this does not seem to be the case. The yeast cell does not turn over the succinic acid under the condition used. Reports from the literature in these questions are collected in the paper mentioned above.

In this case also the permeation rate of the acid was titrimetrically determined for a pure 0.1958 M succinic acid solution. The results of such an experiment are recorded in Table 16. The values in the table are corrected with respect to the dilution with the intercellular water. The potassium emerged from the cell was analysed in the solution with the aid of the flame-spectrophotometer.

Table 16. *Rate of permeation of 0.1985 M succinic acid into the yeast cell.*

Time in min.	% succinic acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
—	—	—	2.55
1	0.8	0.04	2.66
4	0.8	0.05	2.68
8	1.2	0.07	2.69
12	0.5	0.08	2.69
20	0.8	0.11	2.71

At a pH of about 2.6 succinic acid practically does not permeate into the yeast cell, as is shown in Table 16. The probability for a weak acid to permeate is greater the more acid the solution is, because the amount of undissociated acid increases with an increased acidity, and it is only the undissociated form of an acid which can possibly permeate into the cell. From this experiment, therefore, the conclusion can be drawn that succinic acid does not permeate at higher pH than 2.6.

Tartaric acid

Yeast was shaken in a 0.1938 M tartaric acid solution of different pH at 25° C. Samples were removed after 1 and 4 hours. The results are recorded in Table 17. The potassium was analysed in the yeast cells with the aid of the flame-spectrophotometer after incineration of the yeast by the wet process.

Table 17 shows that no potassium loss from the cell occurs in the presence of about 0.2 M tartaric acid at various pH. An exception is, however, found when the yeast has been shaken for 4 hours at an original pH of 2.04.

Table 17. *Effect of 0.1938 M tartaric acid on the potassium content of the yeast cell.*

No.	Time of shaking the yeast cells in tartaric acid				
	0 min.	60 min.		240 min.	
	pH of the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
Control	7	0	—	0	—
1	4.96	0	4.97	0	4.91
2	4.03	0	4.07	0	4.06
3	3.01	0	3.06	0	3.07
4	2.04	0	2.15	7,2	2.19

Table 18. *Rate of permeation of 0.1938 M tartaric acid into the yeast cell.*

Time in min.	% tartaric acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
—	—	—	2.00
1	0.4	0.2	2.05
4	0.4	0.4	2.03
8	0.9	0.6	2.03
12	0.5	0.7	2.03
20	0.5	1.0	2.03

Again an experiment was carried out in order to examine the ability of the pure acid to permeate into the yeast cell, because no quantitative reports could be found in the literature. Samples were taken from a yeast suspension in the pure acid after various times and the acid concentration titrimetrically determined with NaOH in the filtered solution. The samples were also analysed for potassium with the aid of the flame-spectrophotometer. The results are recorded in Table 18.

As shown in Table 18, the permeation ability of tartaric acid at a pH of about 2 is very small. The amount of K which emerged from the cell is also very small. As is to be expected, the pH has not changed.

Citric acid

Yeast was shaken in a 0.2585 M citric acid solution at six different pH. After 1 and 4 hours samples were removed. The control was again prepared in the same way as in the earlier experiments.

Table 19 shows the results of a typical experiment. As can be seen, the content of potassium in the cells in the presence of citric acid has practically not changed during the experiment. The potassium analyses were carried out by the chemical method. The pH-values of the various samples are also practically constant.

According to the investigations of *Wieringa* (1930), citric acid is a little more permeable than tartaric acid. In lack of quantitative reports from the literature, experiments were again carried out in order to determine the permeation rate of the acid. To begin with a 0.2585 M citric acid solution was used. This solution proved to be hypotonic and therefore the concentration of the acid seemed to increase about 7 % after the yeast was shaken in the solution, since the cells had taken up water from the solution. The experiment therefore was repeated with a 0.549 M citric acid solution which proved to be practically isotonic with the yeast cell. The results are recorded

Table 19. *Effect of 0.2585 M citric acid on the potassium content of the yeast cell.*

No.	Time of shaking the yeast cells in citric acid				
	0 min.	60 min.		240 min.	
	pH of the solution	mg.K/100mg. dried yeast	pH of the solution	mg.K/100mg. dried yeast	pH of the solution
Control	7.0	2.12	5.70	2.04	5.78
1	4.96	2.20	4.78	2.16	4.77
2	4.65	2.29	4.48	2.25	4.50
3	4.16	2.23	3.99	1.99	4.00
4	3.69	2.06	3.55	1.99	3.75
5	3.36	—	3.24	2.07	3.28
6	2.97	—	2.90	2.14	2.95

Table 20. *Rate of permeation of 0.549 M citric acid into the yeast cell.*

Time in min.	% citric acid that disappeared from the solution	K, in % of the total cellular K, that escaped from the cell	pH of the solution
—	—	—	1.85
1	0.5	0.97	1.89
4	0.8	1.12	1.87
8	0.8	1.36	1.86
12	0.8	1.65	1.86
20	0.6	2.02	1.86

in Table 20. The filtered solution was also analysed for potassium by the aid of the flame-spectrophotometer.

As is shown in Table 20, citric acid is as little permeable as tartaric acid.

Discussion

The aim of the present investigation was to see if weak acids, which are able to permeate into the cell and which are not turned over by the cell, cause a potassium loss from the cell. The weak acids investigated in the present work are recorded in Table 21 with regard to their ability to permeate into the cell as well as their influence upon the power of the cell to bind potassium.

The acids are arranged after increasing molecule weight in Table 21. The values for the distribution coefficients were partly obtained from the Tables of Landolt-Börnstein, and partly from a paper by Collander (1947). The value for β -hydroxy-butyric acid, which could not be found in the literature, was determined by the author according to a method described by Dermer

Table 21. Comparison of some weak acids, analysed in the present work, with regard to their ability to permeate as well as their influence upon the potassium management of the cell.

Acid	Molecule weight	Dissoc. constant	Distribution coeff. ether/water	Potassium escape	Ability to permeate
Hydrogen fluoride ...	20.0	7.2×10^{-4}	—	Considerable	Very good
Formic acid	46.0	2.1×10^{-4}	0.16	»	» »
Acetic acid	60.0	1.8×10^{-5}	0.5	Slight	» »
Propionic acid	74.1	1.4×10^{-5}	1.7	»	» »
Butyric acid	88.1	1.5×10^{-5}	6.8	»	» »
Isobutyric acid	88.1	1.48×10^{-5}	5.9	»	» »
Lactic acid ..	90.1	1.4×10^{-4}	0.091	None	Is easily turned over by the cell
β -hydroxy-butyric acid	104.1	—	1.27	Slight	Very good
Succinic acid	118.1	6.4×10^{-5}	0.167	Very slight	Poor
Tartaric acid	150.1	9.7×10^{-4}	0.0039	» »	»
Citric acid	192.1	$\left\{ \begin{array}{l} 8.2 \times 10^{-4} \\ 3.2 \times 10^{-6} \end{array} \right\}$	0.065	» »	»

et al. (1941) (conc. of the acid=0.1 M, temp.: 25° C). The acid was obtained from the Bios Laboratories, Inc. New York.

The potassium loss from the cell is indicated in Table 21 as 'considerable', 'slight', or 'very slight'. We have for the present restrained from expressing the potassium loss as a permeability constant. The same applies to the permeation ability. The purpose was instead to make a general survey of the behaviour of a number of acids.

As just mentioned, the permeation ability of the acids is only described and not expressed in quantitative figures. In the case of formic, acetic, propionic, butyric and isobutyric acids, the permeation rate is so rapid that a permeation equilibrium is practically attained after only one minute, too short a time to permit calculations of a permeability constant. To separate the yeast cells from the solution in shorter times than about one minute is impossible. A color-indicator method, such as reported by Colander and Äyräpää (1947) for weak bases, would be desirable for weak acids too. No suitable indicator is, however, known for the acid pH-range.

The weak acids permeate in the undissociated form. The equilibrium conditions require the concentration of the undissociated acid to be equal in the cell and the surrounding medium. Besides the original concentration of the undissociated acid in the medium, which depends on the absolute concentration of the acid concerned as well as on its dissociation constant, the ability of the cell membrane to transmit the acid is also decisive in determining how fast this equilibrium is attained.

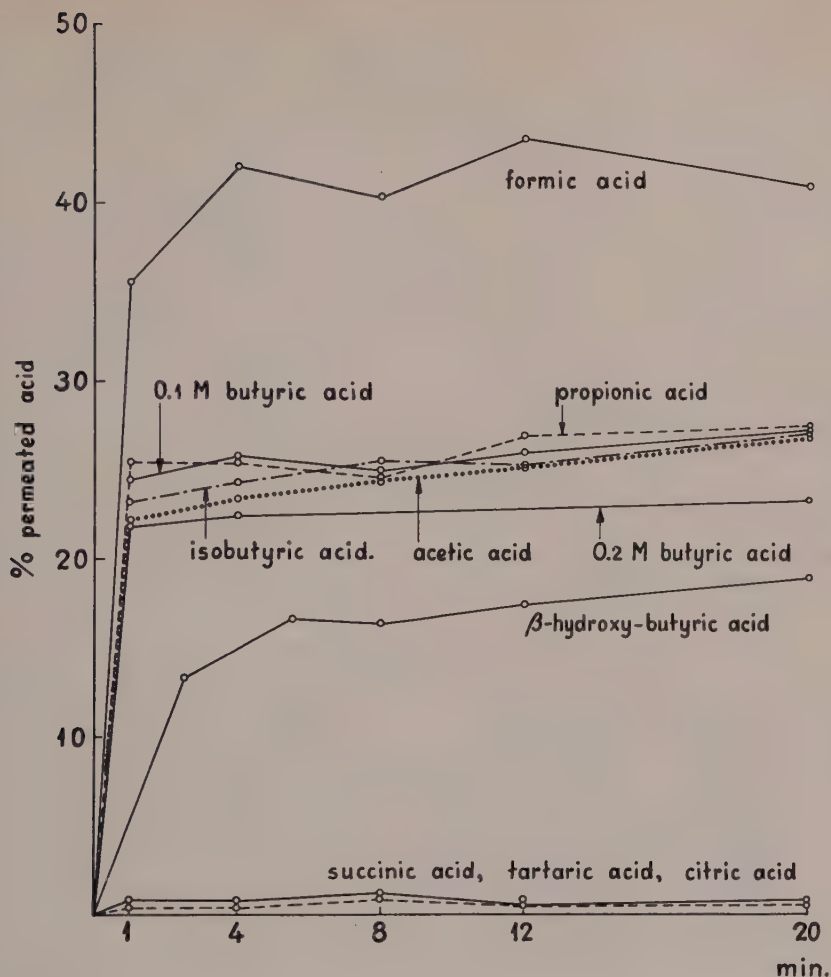


Fig. 1. Rate of permeation of various weak acids into the yeast cell.

A comparison, in Table 21, of the permeation ability and the molecular weight of the acids shows that the permeation ability is relatively good up to a molecular weight of about 90—104.

Of the acids which penetrate well, those with the lowest molecular weight have a marked influence upon the ability of the cell to bind potassium. Thus, for instance, an approximately 0.05 M NaF solution at a pH of 3.0 causes a potassium loss of 60—65 % of the total cellular potassium after one hour (Malm 1947). When yeast is shaken in a 0.1 M formic acid solution at pH 3 sometimes up to 80 % of the cellular potassium has left the cell after 3 hours.

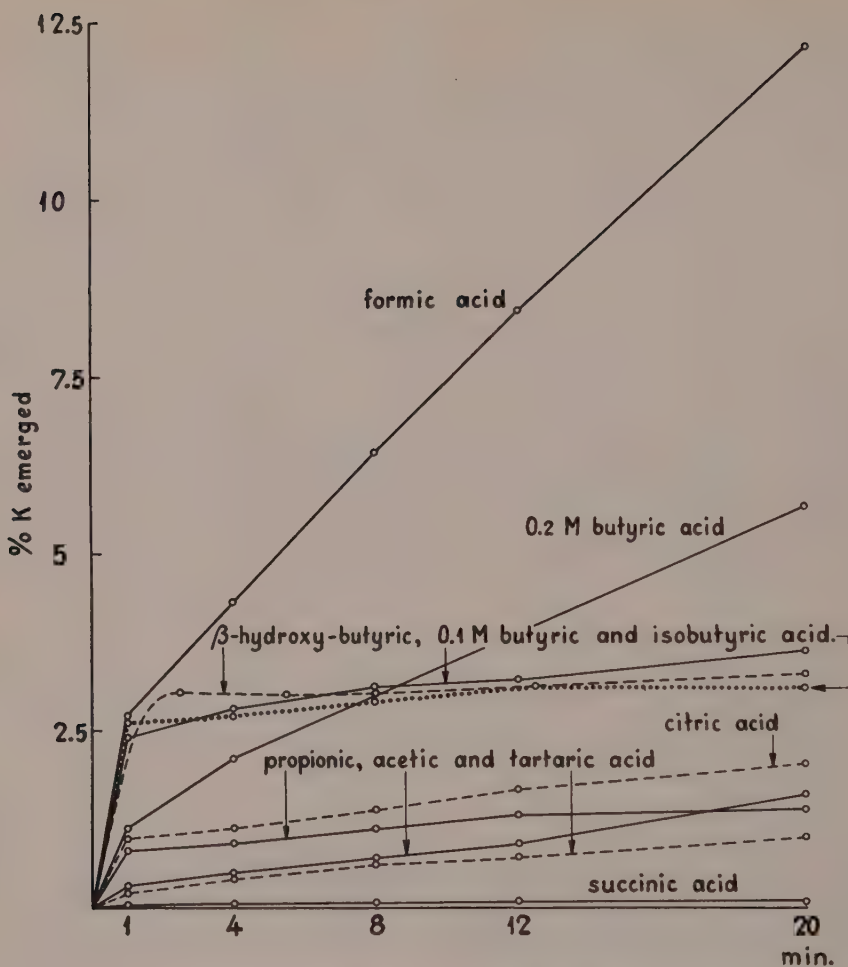


Fig. 2. Rate of the potassium emergence from the yeast cell in the presence of various weak acids.

The four following acids, acetic, propionic, butyric and isobutyric acid, are not inferior to the two first acids with regard to their permeation ability, though the absolute amount of penetrated acid is smaller. The influence of these four acids on the potassium-binding power of the yeast cell is, however, slight. Only at a pH of about 3 or less and at a relatively high concentration is a distinct potassium loss noticed (see Table 10). The same is, on the whole, also valid for β -hydroxy-butyric acid.

Of the remaining acids in Table 21, lactic acid is turned over by the yeast cells. No potassium emerges from the cell in this case.

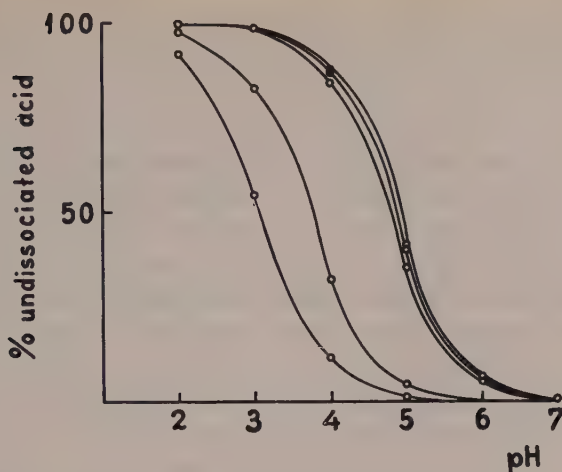


Fig. 3. The dependence of the percentage of undissociated acid of some weak acids upon the pH of the solution. The weak acids are, from left to right: hydrogen fluoride, formic acid, acetic acid, butyric and isobutyric acid, whose curves coincide, and propionic acid.

Succinic, tartaric and citric acid permeate very poorly. The potassium emergence is also very slight.

With the aid of the values in the tables in the preceding section, the rate of penetration of the various acids into the yeast cell is graphically shown in Fig. 1. Fig. 2 shows the rate of the potassium emergence in the presence of the various acids. From a comparison of these two figures it appears that the potassium emergence is not always proportional to the amount of penetrated acid. In spite of the fact that about as much acetic and propionic acid as butyric acid permeates into the cell and that the rate for the permeation is the same, less potassium emerges in the former case. β -hydroxy-butyric acid permeates slower than butyric acid. The amount of the penetrated acid at equilibrium is also smaller in the case of butyric acid. The rate and the magnitude of the potassium emergence, however, is the same.

A cursory survey thus gives the impression that it is not the permeation ability of an acid alone which is decisive for the potassium emergence. If, however, the potassium loss were caused by hydrogen ions, the concentration of the latter, that is, the magnitude of the pH-depression must be decisive for a potassium emergence.

In Fig. 3 it is graphically demonstrated, for the six first acids in Table 21, how the percentage of undissociated acid changes with the pH of the solution. From left to right, the curves correspond to the following acids: hydrogen fluoride, formic acid, acetic acid, butyric and isobutyric acid,

whose curves entirely coincide, and propionic acid. The figures which are the basis of these curves are obtained from the dissociation constants recorded in Table 21.

In an earlier paper (Malm 1947), the author have calculated the pH which prevails in the cell when a distribution equilibrium for hydrogen fluoride of a certain concentration is established between the solution and the cell. Table XIII in the above-mentioned paper shows the calculated pH in the cell at various pH in the medium at equilibrium. The concentration of the hydrogen fluoride used in these experiments was 0.02 M. When the pH of the medium, for instance, was 3.80, the calculated pH of the cell is 4.69.

In the following, the pH in the cell (calculated in the same way as for hydrogen fluoride) at distribution equilibrium for the next 5 acids in Table 21 will be given for certain conditions. Since the author analysed the amount of penetrated acid only in the case when a pure acid solution was used, it was possible to calculate the pH of the cell only at one pH-value in the medium, i.e., the pH of the pure acid at the concentration used.

As an example, the calculation of the pH at distribution equilibrium for formic acid will be given.

From a pure 0.0964 M formic acid solution about 42 % formic acid has penetrated into the cell (see Table 2). The pH of the solution has hereby increased from 2.30 to 2.94. To 3 g. yeast, 10 ml. of a solution containing 44.4 mg. formic acid had been added. Of these 44.4 mg. thus $0.42 \times 44.4 = 18.6$ mg. penetrated into the cell.

Thus, 25.8 mg. formic acid are left in the solution. At pH=2.94, 84 % of the acid is present in the undissociated form, that is $0.84 \times 25.8 = 21.6$ mg. formic acid are undissociated. 3 g. yeast contains 0.78 ml. intercellular water. The volume of the solution has therefore increased to 10.78 ml. The concentration of undissociated formic acid in the solution at distribution equilibrium is then 0.0442 M. The concentration of the undissociated formic acid in the cell should be the same (see p. 394).

The intracellular water of 3 g. yeast is 1.41 ml. From this and from the values mentioned above it can be calculated that 2.8 mg. formic acid are present in the undissociated form in the cell. A total of 18.6 mg. formic acid are present in the cell at equilibrium as mentioned above. 15.1 % are thus present as molecules. From Fig. 4 is seen that 15.1 % undissociated formic acid corresponds to a pH=4.4.

The pH in the cell at distribution equilibrium of the other acids examined has been calculated in the same way as for the formic acid. The pH in the cells of yeast which has been shaken in a 0.1 M acetic acid is 5.03; in a 0.1 M butyric acid solution and isobutyric acid solution 5.17; and in a 0.1 M propionic acid solution 5.20.

The values for the pH in the cell at distribution equilibrium of the various acids are obtained at about the same experimental conditions (concentration and pH of the solution). In the case of hydrogen fluoride, however, an

exception is made. Here the concentration was only $\frac{1}{5}$ of that of the other acids.

As appears from the above, the two first acids in Table 21 cause the lowest pH-values in the cell, viz. 4.69 respectively 4.4. At the same time, 17 respectively 12 % potassium has emerged from the cell. The four following acids do not depress the pH of the cell below pH 5. The potassium emerged from the cell at established distribution equilibrium is kept below 4 % in the case of these four acids.

Of the following acids in Table 21, lactic acid is turned over by the yeast cells as already mentioned. It is not likely that this will depress the pH of the cell. It could rather be expected that the pH would slightly increase thereby. The analysis show that no potassium emergence from the cell takes place.

As the author was not able to find the dissociation constant for β -hydroxybutyric acid in the literature, the pH in the cell could not be calculated in this case.

Succinic, tartaric and citric acid, finally, permeate very poorly, and therefore the pH in the cell will remain unchanged. At the same time, the potassium emergence is very small.

It thus seems as if only permeating acids, which are not turned over by the cell and whose dissociation constant lies about 10^{-4} , cause a strong potassium loss. Weaker acids, on the other hand, are not able to depress the pH in the cell enough to cause a potassium loss. Thus it appears as if acids with a dissociation constant of about 10^{-5} are even too weak. An increase in the concentration of these acids can, however, promote a potassium loss (see p. 386 and Fig. 2).

Independent of each other, dr. H. Ussing and later professor E. J. Conway made the following suggestion in a personal communication. In the case of hydrogen fluoride and formic acid perhaps the anions of these acids, which are accumulated in the cell by the dissociation of the permeated acid, are able to leave the cell together with potassium ions as ion pairs owing to the small size of the ions. The anions of other weak acids, on the other hand, seem already to be too big to allow a permeation back into the solution. This is, however, not easy to prove by experiments. This view could, nevertheless, be supported in the case of hydrogen fluoride by the observation that the fluoride ion concentration sometimes increases again when the suspension is allowed to stand for some time at low pH (see Fig. 24, Malm 1947). The same has also been observed in experiments at anaerobic conditions (see Fig. 35 and 36, Malm 1947). In the case of formic acid, however, it has hitherto not been observed that the acid again emerges from the cell.

Summary

Earlier investigations have shown that potassium emerges from the yeast cell when the latter is shaken in a NaF solution. The potassium emergence seems to be caused by changed electrostatic conditions in the cell. Fluoride penetrates into the cell in the form of undissociated hydrogen fluoride. In the cell, HF dissociates to a large extent into fluoride and hydrogen ions. The more acid the solution surrounding the cells is the more hydrogen ions can be carried into the cells in this way. In an earlier paper, calculations showed that the pH in the cell can hereby be decreased considerably. The changed pH in the cell will surely influence the activity of the enzymes. As a result, non-diffusible anions could be transformed to diffusible ones. In this way potassium, originally electrostatically attracted to non-diffusible anions, is set free and can leave the cell.

If the hydrogen ions were responsible for the potassium emergence, one would expect that other weak acids would also cause a potassium loss from the cell, that is, acids which are able to penetrate into the cell and which are not attacked by the enzymes of the cell. The aim of the present work was to examine the influence of a number of weak acids on the potassium management of the cell.

The following weak acids were examined: formic, acetic, propionic, butyric, isobutyric, lactic, β -hydroxy-butyric, succinic, tartaric and citric acid.

The experiments showed that the permeation ability alone of the weak acids is not decisive for a potassium emergence. It was considered if the dissociation constant of the weak acids was decisive for the potassium emergence. It appeared that only those acids, whose dissociation constant are about 10^{-4} cause a distinct potassium emergence. Acids with a dissociation constant about 10^{-5} seem already to be too weak. An increase in the concentration of these can, nevertheless, promote a potassium emergence.

Independent of each other dr. H. Ussing and later professor E. J. Conway drew the author's attention to the possibility that potassium could perhaps leave the cell together with fluoride or formate ions as an ion pair. The size of the anions of the other acids, on the contrary, does not permit a permeation, and therefore causes no potassium emergence. Investigations on this subject will be presented in a coming paper.

References

- Boyle, P. J. and Conway, E. J.: Potassium accumulation in muscle and associated changes. — *J. Physiol.* 100: 1. 1941.
Brooks, S. C.: Penetration of radioactive ions and their accumulation by protoplasm of living cell. — *Proc. Soc. Exptl. Med.* 38: 356. 1938.

- Collander, R.: On lipid solubility. — *Acta Physiol. Scand.* 13:363. 1947.
- and Äyräpää, T.: A simple permeability experiment for class work and demonstration purposes. — *Acta Physiol. Scand.* 14:171. 1947.
- Conway, E. J.: Exchanges of K, Na and H ions between the cell and its environment. — *Irish J. Med. Sci.* 6:593. 1947.
- and Boyle, P. J.: A mechanism for the concentrating of potassium by cells, with experimental verification for muscle. — *Nature* 144:709. 1939 II.
- and O'Malley, E.: Nature of the cation exchanges during short-period yeast fermentation. — *Nature* 153:555. 1944.
- — Formation of hydrogen ions in high concentration by ordinary baker's yeast. — *Nature* 153:652. 1944.
- Danielli, J. F.: The relations between surface pH, ion concentrations and interfacial tension. — *Proc. Roy. Soc. B* 122:155. 1937.
- The biological action of ions and the concentration of ions at surfaces. — *J. Exp. Biol.* 20:167. 1944.
- Davson, H.: The effect of some metabolic poisons on the permeability of the rabbit erythrocyte to potassium. — *J. cell. comp. Physiol.* 18:173. 1941.
- Dermer, O. C., Markham, W. G. and Trimble, H. M.: Partition ratios of some organic acids between water and ethyl ether. — *J. Amer. Chem. Soc.* 63:3524. 1941.
- Ducleaux, E.: Sur l'action antiseptique de l'acide formique. — *Annales de l'Inst. Pasteur* 6:593. 1892.
- Franzen, H.: Beiträge zur Biochemie der Mikroorganismen, V Mitt. Über die Vergärung und Bildung der Ameisensäure durch Hefen. — *Z. physiol. Chem.* 77:129. 1912.
- Hevesy, G. and Nielsen, N.: Potassium interchange in yeast cells. — *Acta Physiol. Scand.* 2:347. 1941.
- Hevesy, G.: Blodlegemers permeabilitet. — *Forhandl. 5. Nordiska Kemikermøde, København* 1939.
- van Laer, M.: Recherches sur le mode d'action des diastases hydrolysantes. — *Ann. Brass. et Dist.* 19:19. 1920.
- Malm, M.: Quantitative Bestimmungen der Permeabilität der Hefezellen für Fluor. — *Naturwiss.* 28:723. 1940.
- Über den durch NaF verursachten Kaliumaustritt der Oberhefe. — *Arkiv f. Kemi, Miner. o. Geol.* 21 A, no. 4. 1945.
- Über die Permeabilität der Hefezellen und die von den permeierenden Stoffen, insbesondere Fluorwasserstoff, bedingten Plasmaveränderungen. — *Arkiv f. Kemi, Miner. o. Geol.* 25 A, no. 1. 1947.
- Nydahl, F.: Vergleich zwischen einigen Methoden zur Bestimmung des Kalidüngungsbedarfs des Ackerbodens. — *Medd. no. 421 från Centralinst. på Försöksväsendet på Jordbruksområdet, Stockholm* 1932.
- Rothstein, A. and Haeghe Enns, L.: The relationship of potassium to carbohydrate metabolism in baker's yeast. — *J. cell. comp. Physiol.* 28:231. 1946.
- Suomalainen, H.: The role of alpha-glucosidase in the fermentation of oligosaccharides by living yeast. — *Helsingfors* 1948.
- Wieringa, K. T.: Quantitative Permeabilitätsbestimmungen. — *Protoplasma* 8:522. 1930.
- Wilbrandt, W.: A relation between the permeability of the red cell and its metabolism. — *Trans. Faraday Soc.* 33:956. 1937.

On the Base Permeability of Yeast

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Symbols

- a=initial concentration of the base in the reference suspension.
c=total concentration of the base ($[B] + [BH^+]$) in the intracellular phase.
C=total concentration of the base in the medium.
C=molarity (as concentration unit).
d=a relative measure of the molecular length.
I=ionic strength.
k=partition coefficient.
m=mass.
M=molecular weight.
mC=millimolarity.
n=number of carbon atoms in a compound.
P=permeation constant.
 $pH = -\log a_H^+$
 $pK_{(s,b)} = -\log K_{(s,b)}$
q=cell surface area.
r=relative base value (p. 406).
t=time.
T=temperature.
v=volume of the intracellular phase.
V=volume of the suspension medium.
Z=ionic charge.
 α =an empirical factor (p. 412).
 β =base fraction $\left(\frac{[B]}{[B] + [BH^+]} \right)$ in the intracellular phase.
 γ =an empirical factor (p. 413).
 ρ =base fraction in the medium.

I. Introduction

The first investigations on the permeability of plant cells to different bases were made by Overton (1896) and Harvey (1911). They found that weak bases permeate principally as uncharged molecules and that their lipid solubility is a very important factor in their penetration power. The first quantitative experiments to compare the penetration power of different bases were made by Poijärvi (1928). He showed that a very distinct parallelism may be found between lipid solubility and permeation rate, but, on the other hand, the smallest molecules permeate much more rapidly and the largest molecules somewhat more slowly than one would expect from their lipid solubilities.

These authors considered the permeation of weak bases to be a process similar to the permeation of non-electrolytes, i.e. the bases permeate as uncharged molecules, the corresponding cations being practically unable to permeate.

According to Osterhout (1936) and Jacques (1935, 1939) the weak bases form undissociated complexes with the acid compounds of plasma membrane lipoids and these are able to permeate through the membrane. In their experiments the permeation rate was not directly proportional to the concentration gradient of the uncharged base. Therefore they reached the conclusion that the amount of acid components in the plasma membrane is a limiting factor.

The experiments of Osterhout and Jacques were carried out with great accuracy and their method, a direct analysis of *Valonia* cell sap, seems a priori very reliable. But there are other facts which justify a criticism of their theory.

For example, according to Jacques (1939) 20 days were required to reach equilibrium in his experiments with ammonia. He believes that the concentration of uncharged ammonia molecules in the vacuole sap is one-seventh of the corresponding concentration in the medium when equilibrium is reached. But it is evidently an oversimplification to regard this apparent equilibrium («steady state») as a physico-chemical one, depending only on the properties of the plasma membranes. A *Valonia* cell is not just a simple physico-chemical model with semipermeable membranes, but a much more complicated system. The apparent equilibrium, evidently a dynamic one, can be explained on the assumption that the decrease of ammonia concentration for various reasons (possibly by assimilation of ammonium ions, perhaps also by exchange of ions through the membrane) has reached a rate equal to the penetration of ammonia.

Disregarding the other possible sources of error, there is no reason to

generalize from the theory of Osterhout and Jacques. In spite of the possible justification of this hypothesis in cases such as guanidine (Jacques 1935) it is quite clear that uncharged base molecules, having the minimum of kinetic energy to overcome the potential energy barriers of membrane surfaces, can permeate and do so whether they can form complexes with acid membrane compounds or not.

The available facts, as well as theoretical considerations, make it appear probable that the penetration of base-lipoid complexes in many cases may be practically neglected in favour of the penetration of free molecules, particularly when using rapidly penetrating bases and concentration ranges greater than 1 mC. On the other hand, in low concentration ranges (Osterhout 1936) and with bases of very low lipoid solubility (Jacques 1935) the reverse may be the case.

It is possible to investigate whether free molecule penetration preponderates, by examining whether the permeation rate is directly proportional to the concentration gradient. Unfortunately the method of the subsequent investigation is less appropriate for this purpose. Some attempts have been made in very limited concentration ranges (ammonia, Table 3, tests nos. 6257—9, methylamine, p. 410). The results support the theory of free molecule penetration.

The aim of this work is to determine the permeation constants as carefully as possible, particularly with regard to rapidly penetrating bases. There are among these bases some substances with extremely small molecules (ammonia, methylamine), whose permeation velocity is theoretically interesting with a view to obtaining knowledge of the structure of the plasma membrane (cf. Collander 1949 a).

II. Experimental

A. General principles of the method

Baker's yeast (*Saccharomyces cerevisiae*) has been used as the object of the experiments. It is easily available in a uniform standard state and its resistance to the alkaline medium and to the penetrating bases seems very high. The lack of colour and small dimensions of the cells are also technically advantageous characters.

The method is somewhat similar to those used by Harvey (1911) and Poijärvi (1928, Lemna experiments); it has been outlined by Collander and Äyräpää (1947). The yeast is stained with neutral red in a slightly alkaline suspension, after which a more alkaline buffer solution and the base (or corresponding salt) solution are added. Then the penetrating base turns the colour of the yeast cells in the direction of yellow. The time required to

reach a certain yellowish red standard colour is used as an inverse measure of the penetrating power of the base. If then the base content of the cells corresponding to the standard colour, the concentration gradient between the medium and the cell content, and the surface area of the cell can be determined, the permeation constants can be calculated.

The yellowish red colour indicating the end-point of the colour change is obtained suitably by preparing from fresh yeast the so-called reference suspension identical with the other test samples except that it contains only such an amount of a very rapidly penetrating base that the standard yellowish red colour is reached.

As a rough standard in the preparation of the reference suspension the colour of phenol red at pH 7.4, such as is reproduced in the colour chart by Clark (1928), is used.

In this way one can eliminate errors due to the variable acid content of the cells (cf. Conway and O'Malley 1944). The colour comparison can also be made with greater accuracy between two yeast suspensions than between an artificial colour standard and a yeast suspension.

On the other hand a reference suspension containing living yeast only retains a nearly constant colour for a short time ($1/3$ —3 minutes). Therefore it must be prepared separately for each experiment.

Numerous problems arise in connection with the use of such reference suspensions. They are discussed in the following part.

B. Method in detail

1. Reference suspension

The amount of base required to reach the standard colour in the reference suspension depends upon its strength. As it has been found experimentally that the average pH value in the intracellular water phase at this colour is roughly 7.2, it seems that the differences in the amounts of base required are due to the fact that at equilibrium considerable amounts of weaker bases remain in the medium (cf. Poijärvi 1928, p. 64). The base concentration at equilibrium must be the same in the medium as in the intracellular phase. If the base fraction $\left(\frac{B}{[B] + [BH^+]}\right)$ in the medium is designated ρ , its total base concentration $([B] + [BH^+])$ C, and the corresponding quantities in the intracellular phase β and c respectively, the equilibrium can be expressed $\rho C = \beta c$ (Jacobs 1940). This equation shows clearly that even if β is relatively small, the amount of base remaining in the medium may be considerable, especially if ρ is small.

In order to obtain quantitative data on the amounts of bases of different strengths required to reach the yellowish red standard colour the following experiments have been made.

Table 1. *The relative base values of reference bases.*

Base	pK_b	Relative base value; the value of ammonia as unity	Relative base value; the value of iso- amylamine as unity (the r value, cf. p. 406)
Iso-amylamine ...	3.36	0.86 ± 0.06	1.00
Ammonia	4.75	1.00	1.16
Novocaine	5.15	1.55 ± 0.06	1.80
Cocaine	5.59	2.22 ± 0.07	2.58
Thebaine	6.05	2.24 ± 0.11	2.60

Several 5 ml samples of a neutral red stained yeast suspension (3.5 volume % yeast) in a 0.08 C phosphate buffer (pH 11.0—11.2) were prepared. At first there was added to a reference sample the amount of ammonium chloride (usually 0.6—0.8 ml 0.01 C solution) required to reach the standard yellowish red colour. Then to another sample such an amount of a very rapidly penetrating base of the desired strength was added, that the yeast reached exactly the same colour as in the previous sample. This latter procedure is suitably performed in the same way as two-phase titrations. These experiments were repeated with bases of different strengths.

By dividing the quantity of »titrated» base by the amount of ammonia (or any other base) in the reference sample, the so-called relative base value is obtained. The relative base values with the standard errors in titration are shown in Table 1.

These experiments show that the amounts of base required differ relatively little when the bases are stronger than ammonia, yet with regard to weaker bases greater differences appear.

It can be shown that (with the exception of thebaine) almost similar results could be calculated theoretically by assuming the pH value in the intracellular phase to be about 7.2.

These results make it probable that when using a base with $pK_b < 4$ in the reference suspension, practically all the base added to the suspension penetrates into the cells and becomes bound in ionic form there by hydrogen ions.

In other cases, particularly when using reference bases with $pK_b > 5$, a considerable fraction of the base remains in the medium. In order to obtain the fraction penetrating, we must divide the amount added to the reference suspension by the relative base value (the value of iso-amylamine as unity), designated in the following as the r value.

It must be emphasized that these experimental r values are strictly speaking valid only at pH 11.0—11.2. When using a lower pH of the medium the possible error must be evaluated with the aid of the equation $\rho C = \beta c$ (cf. p. 405). With stronger bases ($pK_b < 4$) the r value is practically unity in the pH region higher than 10.0, the correction thus being negligible. But in experiments with ammonia and trimethylamine (Table 4) the correction corresponding to the lower pH values is applied. (The corrections were usually lower than 20 %.)

Because yeast has an astonishing capacity to restore the acid reaction in the cell (cf. Conway and O'Malley 1944) even in solutions of permeable bases, the conditions must be so arranged that the reference base is able to penetrate entirely

into the yeast cells in about 30—60 seconds. When the reference base is strong, it must also have a very high penetration power because the suspension medium should not have too alkaline a reaction, the base fraction thus being far from unity. Therefore iso-amylamine is not ideal as a reference base although it has normally been used. Shortly before the completion of the present study a sample of di-iso-amylamine became available. This almost ideal reference base showed that an error varying concurrently with the base fraction of the medium had been introduced by using iso-amylamine.

A series of experiments (similar to those described on p. 406) have been made to correct this error in terms of the quantity a , indicating the total initial base concentration in the reference suspension. The following values are obtained:

In the pH region	10.50—10.25	the correction factor	0.86 (± 0.03)
» » » »	10.25—10.15	» » »	0.84 (± 0.03)
» » » »	10.15—10.00	» » »	0.81 (± 0.03)

With weaker bases the use of higher base fractions was possible and thus such error became negligible.

2. Buffers and pH measurements

The buffers tested were: 1) disodium phosphate — sodium hydroxide buffer, 2) glycine — sodium hydroxide buffer, 3) borax — sodium carbonate buffer and 4) carbonate — bicarbonate buffer. The carbonate buffer is less appropriate because CO_2 penetrates very quickly. In the borax buffer yeast forms large agglomerations. Similar agglomerations have also been obtained with the glycine buffer; later experiments showed no such effect.

The phosphate buffer was the most employed. It gives homogeneous suspensions but has a low capacity below pH 10.5 in spite of the relatively high ionic strength (0.18—0.24 C). Thus while the base penetrates into the cells, the pH of the suspension medium is shifted towards the acid reaction. This change of pH cannot be neglected. The following method of determining the true pH values during the experiments has been developed: an ordinary test sample of stained yeast suspension is placed in a potentiometer cell with glass and calomel electrodes and a small vigorous stirrer. The solution of base to be examined is quickly injected with a Krogh syringe; simultaneously a stop watch is started. The pH value is read at first at every sixth or seventh second, later every tenth or fifteenth second. Immediately thereafter the ordinary tests to determine the colour change velocity are carried out with identical test samples and with the same base. Then the procedure of pH measurement is repeated; this later test gives generally somewhat lower pH values than the former, because the yeast slightly changes the reaction of the medium. Some typical examples of pH changes are represented in Fig. 1. With the aid of similar graphs for each test the base fractions have been calculated.

The change of pH in the suspension medium depending upon the decrease of base concentration, could also be used to calculate the penetration power of the base. This method, somewhat similar to the method used by Bjerrum (1941) in studying inorganic complex equilibria, has been tried and found to give very satisfactory results. It may be suggested that this method could be very useful in studying the penetration power of weak acids.

The calibration of the pH scale is based upon the pH values of a 0.05 C borax

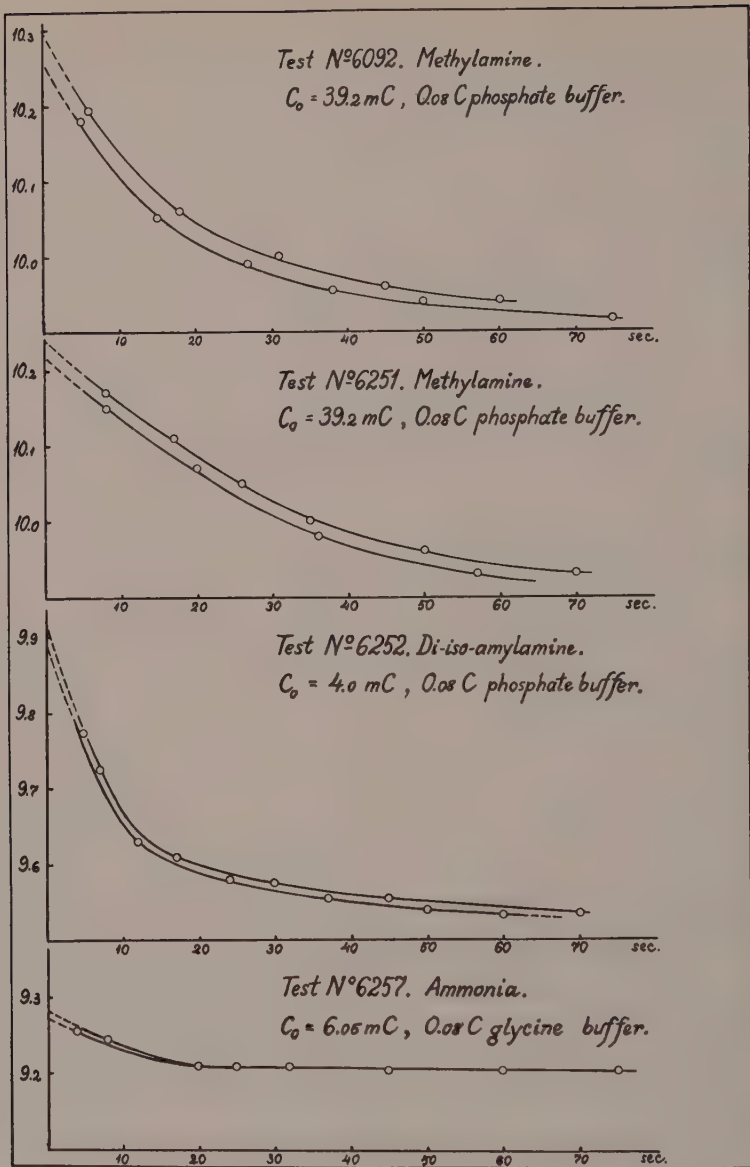


Fig. 1. Some examples of pH change during the experiments. Abscissa: time. Ordinate: pH values (scale readings without sodium correction).

solution, given for various temperatures by Bates (1948, p. 37). The correction needed in the presence of sodium ions is calculated with the aid of a nomogram accompanying the potentiometer used (Radiometer, Type PHM 3h, Copenhagen).

In the work with bases considerably weaker than ammonia the base fraction can be kept near unity; in such cases the pH can be measured directly in the test samples.

3. *Performance of the experiments*

Every day (or once every two days) a sample of 125 g fresh baker's yeast is suspended in 115 ml tap water. The suspension is kept in a refrigerator at $+4^{\circ}\text{C}$. One hour before the experiment takes place it is transferred to room temperature.

The yeast is stained in the following solution (pH ≈ 7.8): 1) 46.7 ml 0.1 $\text{C Na}_2\text{HPO}_4$ solution, 2) 5.0 ml 0.12 % neutral red hydrochloride solution and 3) 13.3 ml yeast suspension, described above.

In this solution the yeast reaches an intense pink-red colour in 5–10 minutes. The neutral red is practically entirely in the intracellular phase (or in lesser degree adsorbed at the cell surface).

The different stages of the experiment are:

1) The pH test: A sample of 6.5 ml is taken from the above-mentioned stained suspension, 2 ml of a buffer solution suitable to reach the desired pH range is added. The sample is placed in a potentiometer cell, 2 ml of the base (or salt) solution in question is injected and the pH change measured as described on p. 407. Simultaneously the time to reach the yellowish red standard colour is presumably estimated.

2) The reference suspension: To a sample of 6.5 ml of the stained suspension is added 2 ml of a convenient buffer and 2 ml of a suitable reference base solution of such a concentration that the yellowish red standard colour is reached. The buffer must be chosen in such a way that the pH of the suspension when ready for use is about the same as in the pH test when it had a similar colour.

3) Measurement of the colour change velocity: 3–5 samples, each containing 6.5 ml of the stained suspension and 2 ml of the same buffer as in the pH test, are prepared. They must be ready at the same time as the reference suspension becomes ready for use. Then 2 ml of the desired base (or salt) solution (the same as in the pH test) is injected into each tube. A stop watch is started at the moment of injection and the time taken to reach the tint of the reference suspension is measured. This procedure must be completed within the time in which the reference suspension has a nearly constant colour.

4) The control pH test is performed in the same way as the first pH test (cf. p. 407).

In certain experiments, the base fraction being nearly unity, exact pH measurements were not carried out; the pH was only roughly checked after the experiments. In these experiments suspensions with a lower yeast content were also used.

C. *Influence of the buffer and the penetrating bases on yeast*

It might be thought that the alkaline suspension medium or the penetrating bases would have an injurious effect upon the yeast cells. In order to examine whether such an effect exists some experiments have been made.

Fink and Kühles (1933) have elaborated a method of separating living and dead yeast cells. By this method four samples have been investigated:

- 1) control test, a sample of yeast stained with neutral red at pH 7.8
- 2) a sample of yeast kept 1 min in 0.02 C atropine solution at pH 11.0
- 3) » » » » 30 sec in 0.02 C iso-amylamine solution at pH 11.0
- 4) » » » » 30 sec in 0.02 C methylamine solution at pH 11.0.

Microscopical check-up showed that the percentage of dead cells in all the samples was practically the same (1.4—1.7 %).

In another experiment samples of yeast were kept for 15 minutes at pH 11.2 1) in a 0.1 C pentamethylene diamine solution, 2) in a 0.1 C tropine solution and 3) in a buffer solution without organic bases. Samples were filtered, made acid (pH 5.6) and after 12 hours suspended in a basic buffer solution (pH 11.0), stained with neutral red and simultaneously made 0.05 molar with tropine. The change of colour occurred in all the samples with the same velocity.

These experiments show that yeast can very well withstand the action of OH^- ions and organic bases. The resistance capacity is also proved by the fact that the stained yeast cells which are made clearly yellow by the penetrating bases turned pink-red again after one or two hours, though the buffer retained its alkaline reaction. However, three hours at pH 11.5 is sufficient to raise the number of dead cells and alter the permeability properties.

According to Ørskov (1945) the permeability of yeast cells to non-electrolytes is strongly influenced by the pH value, at least in acid medium. Unfortunately the method used in the present study only permits comparable experiments within a very limited pH range. But, using a method described on p. 407, it has been found that the significance of pH in the alkaline range is probably not so important. Methylamine showed almost identical permeability constants in the pH range 9.4—10.5 (and with various concentrations of uncharged base molecules, cf. p. 404).

It is also possible that some of the bases examined (especially narcotics and anesthetics), if not directly poisonous, may change the permeability properties of yeast cells. No experiments have been made to examine this possible effect. But on the other hand, the short duration of the experiment probably diminishes any effect of this kind, though it hardly eliminates it. It is also universally known that the growth of moulds and bacteria generally is not influenced by alkaloids.

D. Measurement of the cell surface

The surface area of yeast samples was calculated by using the method of Musfeld (1942). A droplet of yeast suspension in phosphate buffer was examined under the microscope: the length and breadth of 57 cells were measured; results are as shown in Fig. 2. Using Musfeld's graph (i.e., p. 600) the surface area of this cell group is calculated as 4930 sq. μ and the volume as 4200 cu. μ . The yeast samples generally used have a volume of 0.50 cu. cm in tap water and 0.37 cu. cm in the phosphate buffer used (intercellular water excluded). Unfortunately, the volume is not very constant in solutions of relatively high ionic strength (cf. Malm 1947, p. 63 and 68); it can vary within the limits ± 15 %. Thus it is necessary to allow a margin of error of 10 % in the surface area data. The cell surface in normal samples is hence calculated as 3900 ± 400 sq. cm.

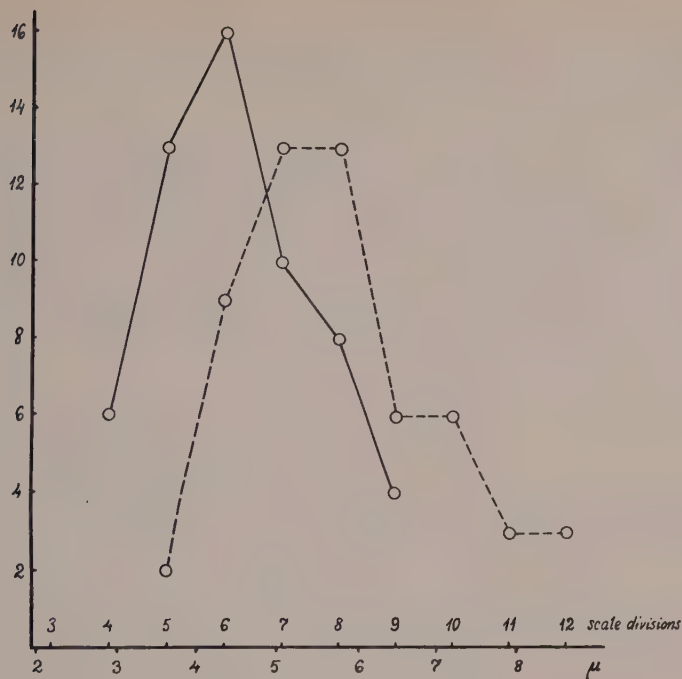


Fig. 2. The variations in the dimensions of yeast cells. Abscissa: dimensions, — cell breadth, ----- cell length. Ordinate: number of cells in each group. (The dimensions expressed in μ and in scale divisions.)

E. Calculation of permeation constants

Fick's diffusion law for penetration through a thin oligomolecular layer is written

$$\frac{dm}{dt} = Pq (C - c) \quad (1)$$

(cf. Collander & Bärlund 1933, p. 25, Davson & Danielli 1943, p. 41, Ørskov 1945, p. 535), where dm is the amount of substance which in the time dt permeates across an area q when the concentration gradient is $C - c$; C is the concentration in the medium, c in the intracellular phase. P is the permeation constant.

For weak electrolytes the concentration gradient can be written $\rho C - \beta c$ (Jacobs 1940, p. 31), where ρ and β are base fractions in the medium and the intracellular phase, respectively.

Substituting $dm = v \cdot dc$ (v is the volume of the intracellular phase) the equation (1) can now be written

$$\frac{dc}{dt} = \frac{Pq}{v} (\rho C - \beta c) \quad (1,1)$$

This equation is exact. But for an exact integration it should be feasible to express the terms C , ρ and β as functions of c (strictly speaking also v and q). For practical purposes certain simplifying approximations are admissible.

As mentioned above (p. 406), in working with bases with $pK_b < 4$, it is admissible to regard all the base that has permeated into the cells as bound by hydrogen ions; thus $\beta c = 0$. Further, provisionally ρ is regarded as a constant. C expressed as a function of c is written $C_0 - \frac{v}{V}c$. C_0 is the initial total base concentration in the medium ($[B] + [BH^+]$) and V the volume of the medium.

Then

$$\frac{dc}{dt} = \frac{Pq}{v} \rho \left(C_0 - \frac{v}{V} c \right) \quad (2)$$

Integrating between the limits 0, c and substituting $\frac{v}{V}c = a$ (a is the initial total base concentration in the reference suspension) we obtain

$$P = \frac{V}{qt\rho} \ln \frac{C_0}{C_0 - a} \quad (2,1)$$

When $a \leq \frac{1}{4}C_0$, we can with sufficient accuracy use an approximate equation, derived from (2) with the trapeze formula:

$$P = \frac{Va}{qt\rho C_m} \quad (2,2)$$

where C_m is the mean value of C_0 and $C_0 - a$.

As mentioned above, the pH changes in the course of the experiment. Thus ρ is not a constant, but a relatively complicated function of c . In order to obtain a practical equation, it is necessary to assume that ρ is a linear function of c . Fig. 3 shows that this approximation is justified: ρ plotted against the decrease of C (which is directly proportional to c) gives a straight line. The experiment is carried out by the method based upon the pH change of the medium (p. 407), and the time taken is twice that required to reach the yellowish red standard colour.

Thus ρ can be written as a function of c : $\rho = \rho_0 (1 - \alpha \frac{v}{V}c)$, where ρ_0 is the initial value and α a constant which can be calculated from the slope of the line ρ plotted against C (in Fig. 3 $\alpha = 0.015$).

When equation (2) is rewritten:

$$\frac{dc}{dt} = \frac{Pq}{v} \rho_0 \left(1 - \alpha \frac{v}{V} c \right) \left(C_0 - \frac{v}{V} c \right) \quad (2,3)$$

Integrating (2,3)

$$P = \frac{V}{qt\rho_0(1 - \alpha C_0)} \ln \frac{C_0(1 - \alpha a)}{C_0 - a} \quad (2,4)$$

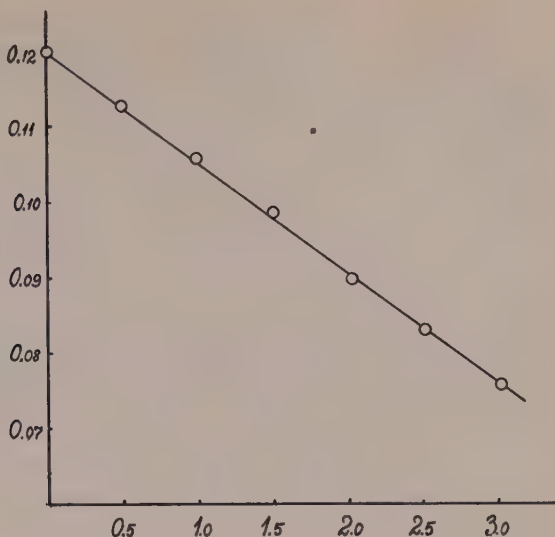


Fig. 3. *Abcissa*: decrease of the total base concentration in the medium. *Ordinate*: base fraction in the medium.

Because the determination of α , if not exactly difficult, still involves a lot of work, the possibility of using the equation (2,1), giving to ρ the mean value of the base fraction during the experiment, may be considered. Comparing the results obtained with equations (2,1) and (2,4) it can be found that the error in all cases is less than 1 %. Thus the equation (2,1) can be regarded as practically correct in the calculation of the permeation constants of the stronger bases ($pK < 4$).

In the case of the weaker bases there are further complications. The concentration gradient is considerably decreased by the increase of the concentration of uncharged base molecules in the intracellular phase. But there are difficulties in expressing the base fraction in the cell (β) in terms of c . A rather practical approximation might be to express β as a linear function of c . (The pH change makes about 1—1.5 pH units — according to Mahdihassan (1930) the pH in the yeast cell vacuole is normally 5.9—6.0 —, and specific experiments have shown that the buffer capacity varies relatively slightly). Then we can write: $\beta = \beta_0 + \gamma c$, where β_0 is the initial value and γ a constant which can be calculated with the aid of β_0 and β_1 (β_1 is the final value).

Putting these values in equation (1,1) we obtain:

$$\frac{dc}{dt} = \frac{Pq}{v} \left[\rho \left(C_0 - \frac{v}{V} c \right) - (\beta_0 + \gamma c) c \right] \quad (3)$$

The integration of (3) results in a relatively complicated equation. Because the final formula would contain many quantities (β , γ , v and c), which must be computed by approximations and assumptions, it is less feasible. How-

ever, a numerical integration has been carried out for the experiment with cocaine (test no. 1061, Table 5). This result has been compared with other possible approximations and it has been found that the most adequate method is a simple equation derived from (3) by the trapeze formula by assuming that the concentration gradient is a linear function of the time. Thus:

$$c = \frac{Pq}{v} \rho \frac{C_0 + (C_0 - a)}{2} t \quad (3,1)$$

In this case $a = \frac{v}{V} c + \frac{\beta_1}{\rho} c$ (valid only if the pH in the reference suspension and in the colour change test are about equal). Then, substituting $\frac{C_0 + (C_0 - a)}{2} = C_m$ in (3,1):

$$P = \frac{vc}{qt\rho C_m}$$

In order to substitute vc by experimentally known quantities it must be noted that in this case only a part of the amount of base a added to the reference suspension, expressed by the symbol $\frac{a}{r}$, has penetrated into the intracellular phase; r is the so-called relative base value (p. 406). Substituting

$vc = \frac{Va}{r}$ we obtain:

$$P = \frac{Va}{rqt\rho C_m} \quad (3,2)$$

However, (3,2) is not practicable, if the concentration gradient decreases by more than 30—35 %.

Equations (2,1), (2,2) and (3,2) are used in the following part; they contain only terms which can be determined experimentally. Certain r values are still corrected by theoretical calculations (experiments with ammonia, Table 4), but the necessary corrections were not large (less than 25 %).

F. Sources of error

1. Systematic errors

a. *General considerations.* When working with bases of various strengths there is some difficulty in estimating the true concentration gradients. The bases have probably to penetrate two plasma membranes, an outer, the plasmalemma and an inner, the tonoplast (cf. Collander 1949 a). As the pH values in the protoplasm between these two membranes cannot be regulated at will, it is fairly obvious that the concentration gradient calculated on the basis of the pH of the medium is not the only one of importance. If the neutral red is accumulated in the vacuole and the colour change thus occurs there, the concentration gradient on the two sides

of the inner membrane is perhaps the most important. Thus the permeation constants for the stronger bases are perhaps relatively too low. As it is difficult to obtain any data to make correct calculations, it must be emphasized that the comparison between values obtained is strictly admissible only if the bases are of the same strength.

It is, however, noteworthy that sparteine (pK_b 2,24) and novocaine (pK_b 5,15), which have a similar ether solubility and an almost identical molecule size, also have similar permeation constants, despite their very different strengths.

b. *The stronger bases* ($pK_b < 4$, Table 3). With the stronger bases the systematic errors principally depend on the fact that the yeast cells tend rapidly to restore the acid reaction of the cell (cf. p. 406). The result is that the term a , using any actual base as reference base, becomes somewhat higher than it would be, using a hypothetical base with infinite penetration velocity. However, it can be shown that by using di-iso-amylamine (permeation constant $8 \cdot 10^{-3}$ cm sec $^{-1}$) this error is negligible (not more than 2–3 %). By using iso-amylamine this error can be corrected, as shown on p. 407. At least it seems probable that the systematic errors in this group amount, at most, to permeation constants 10 % too high.

c. *Ammonia trimethylamine and ephedrine* (Table 4). In this group (pK_b 4–5) the sources of error are the same as in the former group; moreover there is uncertainty over the r values. With regard to the theoretical calculations for obtaining certain r values, the systematic errors may be somewhat higher, but still not greater than 20 %.

d. *The weaker and slowly penetrating bases* (Table 5). In experiments with the weaker basic alkaloids and relatively slowly penetrating bases, the sources of error are still greater. When a long time is required for colour change, the ability of yeast to change the pH in the intracellular phase leads to errors of several tens %. Further, some alkaloids investigated are colloiddally soluble; thereby the estimation of the true concentration gradients becomes very uncertain, especially in the case of atropine, cocaine and thebaine. Kolthoff (1925) gives the following values for their true solubilities in water: atropine $5.5 \cdot 10^{-3}$ C, cocaine $4.0 \cdot 10^{-3}$ C and thebaine $2.2 \cdot 10^{-3}$ C. The concentration of these bases in the experiments (Table 5) was somewhat higher, especially in the experiments with atropine (5–10 times higher). Thus one would expect that in saturated atropine solutions the colour change velocity would have been constant. However, a certain parallelism, but not a direct proportionality, between the atropine concentrations and the colour change velocities has been found in the concentration range used. This phenomenon is perhaps due to the formation of supersaturated solutions and to the fact that the formation of colloidal molecule associations requires a certain time.

The calculation of permeation constants for these bases has been made neglecting these true solubilities, in order not to obtain too high values. Thus these constants represent rough minimum values.

e. *Errors in calculating base fractions*. Base constants determined at 25° C are often used in the calculation of base fractions (ρ), because those valid between 17°–20° C as well as values of the heat of ionization are seldom found in the literature. In the case of bases with smaller molecules (the heat of ionization being generally small) the error due to this fact is negligible. But with bases with larger molecules the error may be somewhat greater; for example in the case of di-iso-amyl- and amylamines it can be estimated to be of the order of 5 %.

The high ionic strength in the phosphate buffer (0.18—0.24 C) brings about an uncertainty in the calculation of the »incomplete» base constants (pk_b) from the thermodynamic constants (pK_b). The calculation is carried out with the aid of the equation

$$\text{pk}_s - \text{pK}_s = (Z_s - \frac{1}{2}) \frac{\sqrt{I}}{1 + \sqrt{I}}$$

properly valid only at ionic strengths lower than 0.1 C. However, the errors due to this fact may be parallel in all cases.

2. Accidental errors

In order to form a quantitative conception of accidental experimental errors, the errors in the terms of equation (2,1) are estimated.

1) V , the volume of the medium, is practically exactly determined.

2) q , the cell surface area, varies considerably (cf. p. 410). An uncertainty of $\pm 10\%$ must be taken into account.

3) t , the time of the colour change, has some uncertainty depending upon the imperfection of the human eye to make exact colour comparisons. By experiments similar to those described on p. 406 this error has been found generally to be lower than $\pm 5\%$ (see also column t in the tables of results).

4) ρ , the base fraction in the medium, cannot be calculated quite exactly. The imperfection in pH measurements (p. 407) brings about an error of, at highest, 5% .

5) C_0 , the initial total base concentration, is practically exactly determined.

The error in a has been explained in the preceding part.

According to a usual rule of error calculation the standard error is then

$$\frac{dP}{P} = \sqrt{\left(\frac{dq^2}{q}\right) + \left(\frac{dt^2}{t}\right) + \left(\frac{d\rho^2}{\rho}\right)} = \sqrt{10^2 + 5^2 + 5^2} \approx \pm 12\%$$

III. Results

A. Tables of results

The bases investigated are given in Table 2.

The results are shown in detail in Tables 3, 4 and 5. In experiments with the stronger bases (Table 3) the following procedure is applied in order to eliminate accidental variations in the permeation constants: Once or twice every day an experiment with methylamine was performed. The other constants obtained on the same day were compared with the constants obtained in the nearest methylamine experiment, and the ratio of the two constants (designated P_n/P_{met}) was calculated. With the aid of the mean values of P_{met} and P_n/P_{met} the reduced permeation constants, P_{red} , were calculated. The experiments mutually compared are to be found with the aid of the experiment number which has the following composition: the first numeral is the month, the second and third are the day and the fourth the current number of the experiment for the date in question.

In Table 4 the same procedure is partially applied, using the permeation constant

Table 2. *The bases investigated with thermodynamic (pK_b) and »incomplete» (pk_b) base constants used.*

Base	Origin	pK_b	pk_b	temp.	Author
Ammonia	Schering	4.75	$\begin{cases} 4.60 \\ 4.66 \end{cases}$	25°	Harned & Owen (1930)
Hydrazine	»	6.04	—	18°	Schwarzenbach (1936)
Methylamine	Eastman-Kodak	3.36	3.20	25°	Harned & Owen (1930)
Dimethylamine	B.D.H.	3.28	3.12	25°	» » »
Trimethylamine	Eastman-Kodak	4.26	4.10	25°	» » »
Ethylamine	B.D.H.	3.33	3.17	25°	Hall & Sprinkle (1932)
Diethylamine	Kebo	3.02	2.86	25°	» » »
Triethylamine	Schering	3.25	3.09	25°	Harned & Owen (1930)
Iso-amylamine	Schuchardt	3.36	3.20	25°	Hall & Sprinkle (1932)
Di-iso-amylamine	Wenner-Gren Inst. (dept of techn. chem.)	2.99	2.83	25°	» » »
Ethanolamine	Schering	4.56	4.40	25°	» » »
Diethanolamine	»	6.05	—	25°	» » »
Diethylethanolamine	»	—	4.45	18°	Äyräpää (unpublished)
Atropine	Pharm. prod.	—	4.35	15°	Kolthoff (1925)
Cocaine	» »	—	5.59	15°	» » »
L-Ephedrine	Merck	4.53	4.37	17.5°	Äyräpää (1950)
Novocaine	Pharm. prod.	—	5.15	15°	Kolthoff (1925)
Sparteine	Merck	—	2.24	15°	» » »
Thebaine	Pharm. prod.	—	6.05	15°	» » »

for ammonia (P_{amm}) as a standard value. Because these experiments were carried out at a lower pH, a direct comparison between the values in Tables 3 and 4 has not been made.

An analogous reduction in Table 5 was not performed owing to the low experimental accuracy. Because these experiments were carried out in a higher pH region, a direct comparison between the values in Tables 4 and 5 is less exact. However, the constants for ephedrine and atropine show that very great differences probably do not exist.

The calculations have been carried out with an accuracy of three numerals; the results are given with two. The second numeral is still often uncertain.

The natural dimension of the permeation constant is cm sec^{-1} (Rashevsky & Landahl 1940); it has consequently been applied.

It must be emphasized that the permeation constants calculated indicate the diffusion resistance of the whole yeast cell. Now it might be thought that the resistance of the cell wall may have an influence on the penetration velocity of the most rapidly penetrating bases. But there is a fact which indirectly proves that such an effect probably does not exist: the permeation constant of di-iso-amylamine is about 30 times higher than the constant of iso-amylamine, roughly corresponding to the difference in the relative ether solubility (cf. Table 6).

The individual variations of permeation constants are not much greater than those found by other more accurate methods (cf. Collander & Bärlund 1933, p. 55). But, on the other hand, the relatively great variations of the quotient P_n/P_{met} makes it probable that there are perhaps greater accidental errors than those calculated on p. 416.

Table 3. *Stronger bases.* Equations applied (2,1) and (2,2). In all experiments $V = 10$ cu. cm and $q = 3900$ sq. cm. Concentrations (C_0 and a) expressed in mC, permeation constants in cm sec^{-1} (cf. p. 416).

No.	T°C	Base	pH	ρ	C_0	a	t_{sec}	P_n	P_n/P_{met}	P_{red}
2051	20°	Methylamine	10.09	0.116	41.6	1.30	12.9 ± 0.3	$5.50 \cdot 10^{-5}$		
2052	20°	»	10.26	0.163	41.6	1.38	9.4 ± 0.2	$5.70 \cdot 10^{-5}$		
2071	18°	»	10.21	0.126	41.6	1.38	11.8 ± 0.3	$5.86 \cdot 10^{-5}$		
2072	18°	»	10.03	0.087	41.6	1.30	14.4 ± 0.2	$6.55 \cdot 10^{-5}$		
2101	19°	»	10.30	0.163	40.0	1.38	9.1 ± 0.3	$6.13 \cdot 10^{-5}$		
2112	20°	»	10.37	0.200	40.0	1.38	7.0 ± 0.1	$6.48 \cdot 10^{-5}$		
2122	19°	»	10.34	0.176	40.0	1.38	7.2 ± 0.0	$7.17 \cdot 10^{-5}$		
2142	19°	»	10.17	0.126	40.0	1.38	8.0 ± 0.3	$9.01 \cdot 10^{-5}$		
2174	21°	»	10.16	0.145	40.0	1.30	7.4 ± 0.3	$7.95 \cdot 10^{-5}$		
6092	18°	»	10.31	0.154	39.2	1.38	7.5 ± 0.3	$8.03 \cdot 10^{-5}$		
6102	18°	»	10.19	0.121	39.2	1.38	9.2 ± 0.4	$8.32 \cdot 10^{-5}$		
6112	17.5°	»	10.22	0.124	39.2	1.38	9.9 ± 0.4	$7.56 \cdot 10^{-5}$		
6114	17.5°	»	10.20	0.119	39.2	1.38	10.8 ± 0.9	$7.22 \cdot 10^{-5}$		
6121	17.5°	»	10.21	0.121	39.2	1.38	11.4 ± 0.4	$6.71 \cdot 10^{-5}$		
6123	17.5°	»	10.23	0.126	39.2	1.38	10.9 ± 0.2	$6.80 \cdot 10^{-5}$		
6131	17.5°	»	10.22	0.124	39.2	1.38	12.4 ± 0.4	$6.03 \cdot 10^{-5}$		
6133	17.5°	»	10.29	0.142	39.2	1.38	9.2 ± 0.2	$7.10 \cdot 10^{-5}$		
6221	17°	»	10.34	0.154	39.2	1.60	10.8 ± 0.6	$6.45 \cdot 10^{-5}$		
6223	17°	»	10.37	0.163	39.2	1.60	10.3 ± 0.4	$6.40 \cdot 10^{-5}$		
6251	17°	»	10.25	0.129	39.2	1.60	14.5 ± 0.6	$5.75 \cdot 10^{-5}$		
6256	17°	»	10.32	0.148	39.2	1.60	12.6 ± 0.6	$5.78 \cdot 10^{-5}$		
								$6.78 \cdot 10^{-5}$		$6.8 \cdot 10^{-5}$
6093	18°	Dimethylamine	10.32	0.134	42.4	1.38	11.4 ± 0.2	$5.58 \cdot 10^{-5}$	0.70	
6115	17.5°	»	10.21	0.103	42.4	1.38	14.5 ± 0.5	$5.72 \cdot 10^{-5}$	0.77	
								0.73		$5.0 \cdot 10^{-5}$
2113	20°	Ethylamine	10.17	0.126	72.5	1.34	11.3 ± 0.2	$3.40 \cdot 10^{-5}$	0.52	
6101	18°	»	10.25	0.126	40.4	1.38	31.1 ± 2.0	$2.30 \cdot 10^{-5}$	0.28	
6103	18°	»	10.24	0.124	40.4	1.38	24.5 ± 0.8	$2.92 \cdot 10^{-5}$	0.35	
6222	17°	»	10.35	0.145	39.9	1.60	30.0 ± 1.0	$2.42 \cdot 10^{-5}$	0.38	
								0.38		$2.6 \cdot 10^{-5}$
2053	20°	Diethylamine	10.52	0.140	16.0	1.38	8.8 ± 0.4	$1.89 \cdot 10^{-4}$	3.31	
2111	19°	»	10.27	0.077	16.0	1.38	16.2 ± 0.4	$1.87 \cdot 10^{-4}$	2.90	
6122	17.5°	»	10.57	0.126	19.7	1.38	15.5 ± 0.5	$9.60 \cdot 10^{-5}$	1.42	
6132	17.5°	»	10.51	0.112	19.7	1.38	18.6 ± 0.6	$9.02 \cdot 10^{-5}$	1.37	
								2.25		$1.5 \cdot 10^{-4}$
2103	20°	Triethylamine	10.41	0.176	40.0	1.38	10.9 ± 0.3	$4.73 \cdot 10^{-5}$	0.77	
2114	19°	»	10.16	0.099	40.0	1.38	14.1 ± 0.4	$6.54 \cdot 10^{-5}$	1.01	
2173	21°	»	10.18	0.121	40.0	1.34	10.1 ± 0.3	$7.25 \cdot 10^{-5}$	0.91	
6111	17.5°	»	10.21	0.097	41.0	1.38	16.9 ± 0.6	$5.44 \cdot 10^{-5}$	0.72	
6113	17.5°	»	10.19	0.093	41.0	1.38	18.0 ± 0.2	$5.16 \cdot 10^{-5}$	0.72	
								0.83		$5.7 \cdot 10^{-5}$
2121	19°	Iso-amylamine	10.34	0.176	8.00	1.38	9.3 ± 1.7	$3.01 \cdot 10^{-4}$	4.2	
6091	17.5°	»	10.18	0.113	8.00	1.38	12.2 ± 0.7	$3.58 \cdot 10^{-4}$	4.45	
6124	17.5°	»	10.41	0.179	8.00	1.38	10.2 ± 0.5	$2.70 \cdot 10^{-4}$	4.0	
6134	17.5°	»	10.42	0.183	8.00	1.38	8.6 ± 0.5	$3.13 \cdot 10^{-4}$	4.4	
								4.26		$2.9 \cdot 10^{-4}$
6252	17°	Di-iso-amylamine	9.89	0.0268	4.00	1.60	6.5 ± 0.6	$7.60 \cdot 10^{-3}$	130	
6254	17°	»	10.02	0.0371	4.00	1.60	5.8 ± 0.4	$6.15 \cdot 10^{-3}$	106	
								118		$8.0 \cdot 10^{-3}$
6224	17°	Sparteine	10.91	0.069	12.5	1.60	12.7 ± 0.6	$3.71 \cdot 10^{-4}$	5.8	$3.9 \cdot 10^{-4}$

Table 4. *Ammonia, trimethylamine, ephedrine and atropine*. V , q and units as in Table 3. Equation used (3,2) (cf. p. 416).

No	T° C	Base	pH	ρ	C_o	a	r	t_{sec}	P_n	P_n/P_{amm}	P_{red}
6151	17.5°	Ammonia	9.54	0.425	4.04	1.60	1.34	11.2 ± 0.3	$2.06 \cdot 10^{-4}$		
6153	18°	»	9.53	0.431	4.04	1.60	1.35	9.7 ± 0.3	$2.31 \cdot 10^{-4}$		
6155	17.5°	»	9.64	0.483	4.04	1.60	1.34	9.1 ± 0.2	$2.17 \cdot 10^{-4}$		
6161	17.5°	»	9.17	0.240	6.05	1.60	1.44	8.2 ± 0.2	$2.78 \cdot 10^{-4}$		
6163	17.5°	»	9.17	0.240	6.05	1.60	1.44	8.2 ± 0.3	$2.78 \cdot 10^{-4}$		
6165	17.5°	»	9.39	0.344	6.05	1.60	1.50	5.4 ± 0.4	$2.82 \cdot 10^{-4}$		
6255	17°	»	9.32	0.338	6.05	1.60	1.33	7.6 ± 0.3	$2.30 \cdot 10^{-4}$		
6257	17°	»	9.27	0.313	6.05	1.60	1.33	7.5 ± 0.1	$2.52 \cdot 10^{-4}$		
6258	17°	»	9.55	0.465	6.05	1.60	1.26	4.9 ± 0.1	$2.74 \cdot 10^{-4}$		
6259	17°	»	9.02	0.200	6.05	1.60	1.41	10.2 ± 0.7	$2.74 \cdot 10^{-4}$	—	$2.5 \cdot 10^{-4}$
6154	17.5°	Trimethylamine	9.12	0.081	60.6	1.60	1.34	33 ± 2	$1.96 \cdot 10^{-5}$	0.086	
6166	17.5°	»	9.34	0.129	67.5	1.60	1.30	14.6 ± 0.5	$2.58 \cdot 10^{-5}$	0.092	
6152	17.5°	l-Ephedrine	9.46	0.300	20.0	1.60	1.26	18.4 ± 1.7	$3.10 \cdot 10^{-5}$	0.089	$2.2 \cdot 10^{-5}$
6162	17.5°	Atropine	9.59	0.323	44.4	1.60	1.35	29 ± 1	$7.46 \cdot 10^{-6}$	0.142	$3.5 \cdot 10^{-5}$
6211	17°	»	10.27	0.690	22.2	1.60	1.0	110	$2.46 \cdot 10^{-6}$	0.027	$6.8 \cdot 10^{-6}$
	17°	»	9.70	0.375	44.4	1.60	1.0	55	$4.50 \cdot 10^{-6}$	With regard to these values see p. 415.	
	17°	»	9.38	0.224	44.4	1.60	1.0	65	$6.40 \cdot 10^{-6}$		

Table 5. *Weaker bases*. Units as in Table 3. All the experiments performed at pH 11.0–11.2, thus $\rho \sim 1$. Equation used (3.2) (cf. p. 416).

No.	T° C	Base	q	V	C_o	a	r	t_{sec}	P_n	Mean values
1193	17°	Hydrazine	1100	4.1	37	1.35	2.60	92 \pm 8	5.7 $\cdot 10^{-7}$	5.7 $\cdot 10^{-7}$
1012	19°	Ethanolamine	1360	5.1	98	0.70	1.17	76 \pm 5	3.0 $\cdot 10^{-7}$	2.0 $\cdot 10^{-7}$
1231	16°	»	1360	5.1	490	0.75	1.17	28.5 \pm 2	1.72 $\cdot 10^{-7}$	
1232	16°	»	1360	5.1	490	1.0	1.17	50 \pm 4	1.31 $\cdot 10^{-7}$	1.7 $\cdot 10^{-6}$
1011	19°	Diethylethanolamine	1360	5.1	20	0.70	1.17	66 \pm 5	1.72 $\cdot 10^{-6}$	
1051	18°	L-Ephedrine	1360	5.1	4.0	0.72	1.17	22 \pm 2	3.40 $\cdot 10^{-5}$	3.4 $\cdot 10^{-5}$
1062	17°	Novocaine	1250	4.8	4.15	1.16	1.80	4.2 \pm 0.3	1.64 $\cdot 10^{-4}$	
1064	17°	»	1250	4.8	4.15	1.16	1.80	4.2 \pm 0.4	1.64 $\cdot 10^{-4}$	1.6 $\cdot 10^{-4}$
1052	18°	Atropine	1360	5.1	19.7	0.75	1.17	22 \pm 2	5.6 $\cdot 10^{-6}$	4.5 $\cdot 10^{-6}$
1063	17°	»	1360	5.1	20.7	0.75	1.17	36 \pm 2	3.3 $\cdot 10^{-6}$	
1061	17°	Cocaine	1250	4.8	4.65	1.66	2.58	11.0 \pm 0.5	5.9 $\cdot 10^{-5}$	5.9 $\cdot 10^{-5}$
1181	18°	Thebaine	1100	4.1	4.90	1.35	2.60	39 \pm 2	1.45 $\cdot 10^{-5}$	1.2 $\cdot 10^{-5}$
1191	18°	»	1100	4.1	4.90	1.35	2.60	41 \pm 4	1.4 $\cdot 10^{-5}$	
1192	17°	»	1100	4.1	4.90	1.35	2.60	43 \pm 2	1.3 $\cdot 10^{-5}$	
1211	17°	»	1100	4.1	4.90	1.35	2.60	70 \pm 5	8.1 $\cdot 10^{-6}$	
1182	30°	»	1100	4.1	4.90	1.35	2.60	8 \pm 1	7.1 $\cdot 10^{-5}$	—
1184	30°	Diethanolamine	1100	4.1	200	1.35	2.60	150 \pm 16	6.5 $\cdot 10^{-8}$	— ¹

¹ If the temperature coefficients for thebaine and diethanolamine are of the same order of magnitude, the P value of diethanolamine at 18° is about $1.3 \cdot 10^{-8}$

Table 6. *Molecular weights (M), logarithms of partition coefficient ether/water (k_{et}), permeation constant (P) and product $PM^{\frac{1}{2}}$.*

Base	M	log k_{et}	log P	log $PM^{\frac{1}{2}}$
Di-iso-amylamine	157	2.59	— 2.10	— 1.09
Cocaine	303	2.12	— 4.2	— 3.0
Sparteine	234	1.82	— 3.41	— 2.23
Novocaine	236	1.59	— 3.8	— 2.6
Thebaine	313	1.20	— 4.9	— 3.7
Iso-amylamine	87	0.83	— 3.54	— 2.57
L-Ephedrine	165	0.77	— 4.46	— 3.35
Atropine	289	0.61	— 5.2	— 4.0
Triethylamine	101	0.30	— 4.24	— 3.24
Diethylamine	73	— 0.27	— 3.82	— 2.89
Diethylethanolamine	117	— 0.46	— 5.8	— 4.7
Trimethylamine	59	— 0.46	— 4.66	— 3.77
Ethylamine	45	— 1.22	— 4.58	— 3.75
Dimethylamine	45	— 1.26	— 4.30	— 3.47
Methylamine	31	— 1.64	— 4.17	— 3.42
Ammonia	17	— 2.15	— 3.60	— 2.98
Diethanolamine	105	— 2.27	— 7.9	— 6.9
Ethanolamine	61	— 2.89	— 6.7	— 5.8
Hydrazine	32	< — 4.0	— 6.2	— 5.5

B. *Relation between the lipid solubility and the penetration power of bases*

Because there are no data available about the partition of bases in the system membrane lipid/water it is necessary to use partition coefficients in model systems.

Collander (1947, 1949 b, 1950) has determined the partition coefficients in the systems ether/water and iso-butanol/water. As the former coefficients (designed k_{et}) of most organic compounds show a very close parallelism to coefficients in the system olive oil/water, they have been employed.

In Table 6 the bases are placed after their relative ether solubilities. The permeation constants and some other data needed in the following discussion are also noted.

The relation between penetration power and lipid solubility becomes more obvious if these data are arranged in a customary logarithmic graph, plotting the product of the permeation constant (P) and the square root of the molecular weight ($M^{\frac{1}{2}}$) (according to Davson & Danielli 1943) against the partition coefficient ether/water. Fig. 4 shows a distinct correlation between penetration power and lipid solubility. With the exception of some too low or uncertain values, the points falling outside the dotted lines are only those of the smallest molecules ($M < 50$) and diethylamine. The apparently too high penetration power of diethylamine obtains an explanation from a theory of Wartiovaara's (see below p. 424).

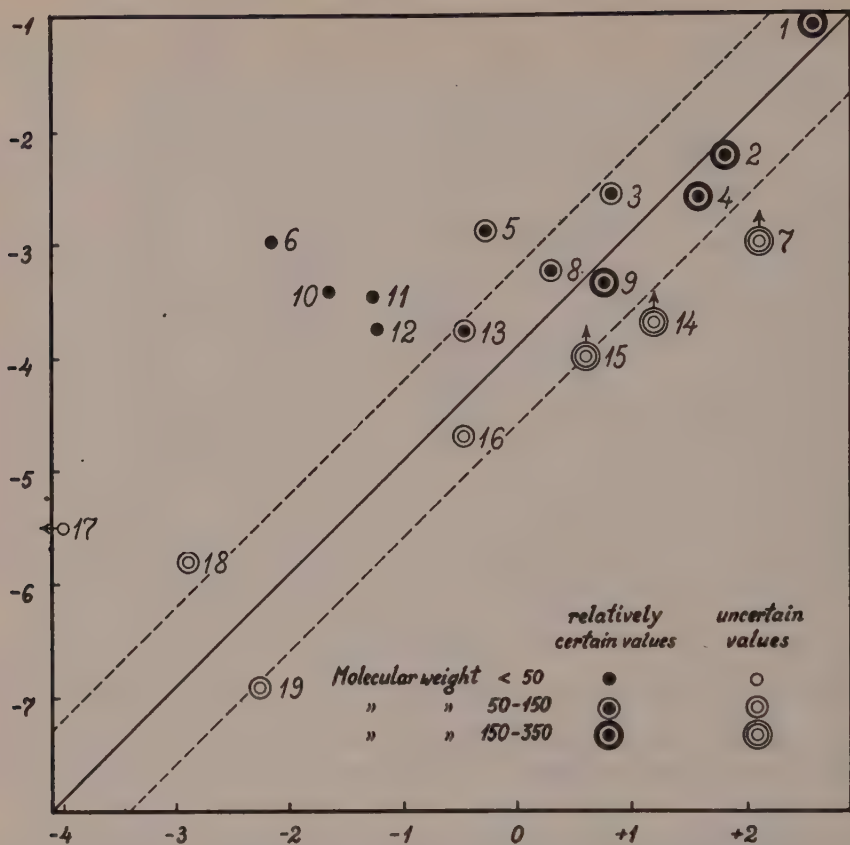


Fig. 4. Abscissa: $\log k_{et}$. Ordinate: $\log PM^{1/2}$. The dotted lines represent values 5 times greater or smaller than the average. Arrows indicate values which are too low (cf. p. 415).

The points are:

- | | | |
|---------------------|--------------------|-------------------------|
| 1) Di-iso-amylamine | 8) Triethylamine | 15) Atropine |
| 2) Sparteine | 9) Ephedrine | 16) Diethylethanolamine |
| 3) Iso-amylamine | 10) Methylamine | 17) Hydrazine |
| 4) Novocaine | 11) Dimethylamine | 18) Ethanolamine |
| 5) Diethylamine | 12) Ethylamine | 19) Diethanolamine |
| 6) Ammonia | 13) Trimethylamine | |
| 7) Cocaine | 14) Thebaine | |

The deviations of the quotient $PM^{1/2}/k_{et}$ for ammonia and methylamine are too great (about 1000 and 125) to be explained on the grounds of shortcomings in the experiments; the same is to some extent true also as regards ethylamine, dimethylamine and hydrazine.

The yeast cell thus shows a very distinct sieve-effect. The smallest mole-

cules (up to molecular weight 50—75) permeate much more rapidly than somewhat larger molecules of corresponding lipid solubility. This is in fair accordance with the results of Poijärvi (1928). There is, however, no evidence that the largest molecules penetrate relatively too slowly; the differences fall within the limits of experimental accuracy.

C. *Explanation of the sieve-effect of the plasma membrane*

Danielli (Davson & Danielli 1943) has developed a theory which attempts to take into account some of the structure of the system through which the penetration occurs. According to this theory for rapidly penetrating molecules the quotient $PM^{1/2}/k$ and for slowly penetrating molecules $PM^{1/2}e^{2500x/RT}/k$ should be approximately constant (l.c., p. 352). Danielli applied these formulae to results of Collander & Bärlund (1933) and Marklund (1936) and claimed to have found a certain accordance with the theory. However, the permeation constants for the most rapidly penetrating substances in these works were rather approximate minimum values. Recently Collander (1949 a) has criticized these conclusions of Danielli.

Furthermore, as pointed out by Danielli (Davson & Danielli 1943, p. 81), the theory is strictly speaking valid only for more or less spherical molecules.

For all the bases investigated in the present study the former formula ($PM^{1/2}/k \sim \text{constant}$) would be applied. As seen from Fig. 4 the discrepancy between the results in this study and the theory of Danielli is very marked.

Thus it seems evident that Danielli's theory cannot be valid for threadlike or rod-shaped molecules, such as particularly primary amines.

Wartiovaara (1949) explains the apparent sieve-effect of the plasma membrane in the following way: In a homologous series, with increasing length of molecules orientation becomes concurrently a more and more important factor which reduces the probability that a molecule with sufficient kinetic energy will actually penetrate. Wartiovaara has made experiments on the permeability of Nitella to normal primary alcohols and found that a close correlation exists between the quotient P/k and the transverse moment of molecular inertia. An equal correlation can be found between $PM^{1/2}/k$ and the magnitude $1/n^2$ (n =number of carbon atoms of the permeating compound). (See also Wartiovaara 1950).

Wartiovaara has made his experiments at different temperatures and found almost equal temperature coefficients for different alcohols. This proves that the explanation of the sieve-effect may be sought in a statistical factor. Then the orientation factor is the only possibility if we suppose that Danielli has taken all the factors into account in his theory.

As the temperature coefficients of the yeast cell permeability are still unknown, we cannot be sure that the sieve-effect exhibited by the yeast cell membrane could be explained in the same way as that of *Nitella*. But, on the other hand, there are some results which offer a rather interesting opportunity of testing the validity of Wartiovaara's hypothesis. The permeation constants of alkylamines, showing great differences in penetration power (P constant of di-iso-amylamine is about 500 times greater than that of ethylamine) are suitable for more detailed examination.

It must be emphasized that the significance of the orientation factor may be different in the groups of primary, secondary and tertiary amines. As the form of tertiary amine molecules is virtually different from the other groups, they have not been compared with the other amines.

Furthermore, it is not possible to use n^2 as a relative measure of the transverse moment of molecular inertia, because many of the amines investigated have branched molecules. Instead another approximative expression is used, d^2n , where d is the distance between the nitrogen atom and the center of gravity of the hydrocarbon group and n the number of carbon atoms. The magnitude of d can easily be calculated from structural data.

The molecular inertia for the ammonia molecule cannot be calculated in a similar way. But as a rough approximation we can use a value which is about ten times less than that of methylamine. The importance of the orientation factor in the penetration of ammonia might also be diminished by the fact that the nitrogen atom in the ammonia molecule vibrates between two positions.

In order to visualize the importance of lipid solubility in Fig. 5, $\log Pd^2n$ is plotted against $\log k_{et}$. The correlation between these two related quantities is at least as close as might be expected on the basis of the experimental accuracy. Evidently the spreading of points depends partially upon the fact that the amines investigated represent two different series for which d^2n and perhaps also k_{et} have not an equivalent significance. The upper and lower diagonal lines in Fig. 5 represent the average lines for primary amines and ammonia, and secondary amines, respectively.

The hypothesis of Wartiovaara is thus able to explain the sieve-effect of the yeast plasma membrane, at least with regard to the penetration of more or less rod-shaped molecules.

The explanation of the fact that the tertiary amines permeate more slowly than both primary and secondary amines of similar lipid solubilities cannot be given on the basis of the results of this study. It can be suggested that the activation energy needed is greater, yet the answer cannot be given until an examination of temperature coefficients has been made.

Ørskov (1945) has investigated the permeability of yeast cells to non-electrolytes and believes that some of them permeate through pores and others across the lipid

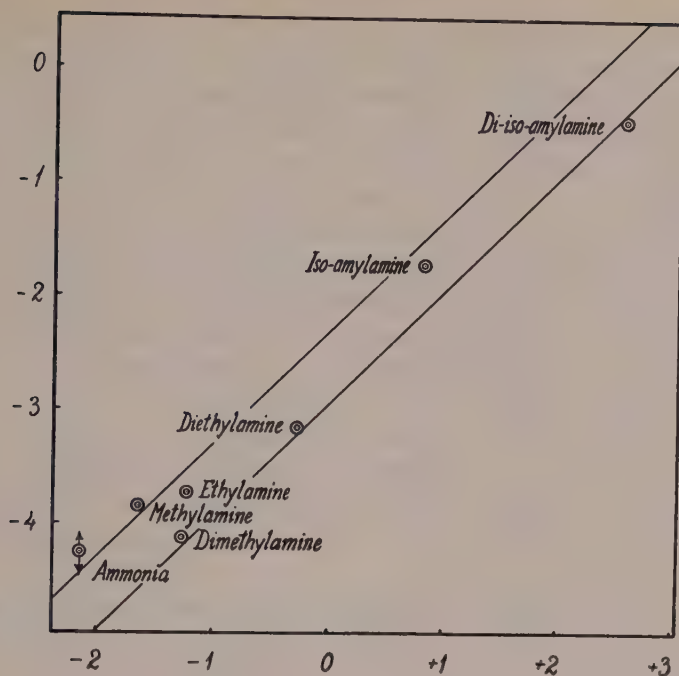


Fig. 5. Abscissa: $\log k_{et}$. Ordinate: $\log Pd^2n$.

layer. But in his choice of substances the largest molecules are generally also the less lipid soluble. Antipyrine alone represents the theoretically important lipid soluble and high molecular compound group.

A critical scrutiny of Ørskov's results, taking into account the influence of the orientation factor, proves that there are no reasons to maintain the pore theory.

Thus, actually, the permeability properties of the yeast cell may be principally explained by assuming that the plasma membrane is homogeneous in a first approximation, i.e. that there is no evidence for the existence of water filled pores. But, on the other hand, it has yet to be proved whether the discontinuities in the plasma membrane have any importance for the penetration of the smallest molecules (cf. Collander & Wikström 1949). There, also, the examination of the temperature coefficients should be fruitful.

D. Permeability of the yeast cell in relation to some other cell types

A direct comparison between the permeability properties of the yeast cell and other cell types is possible only to a very limited extent. In the investigations hitherto made on base permeability, absolute permeation constants (i.e.

cell surface and volume taken into account) have seldom been obtained. A comparison between bases and non-electrolytes, on the other hand, can easily lead to erroneous conclusions, because the penetration velocity may change with pH (cf. Ørskov 1945) and because the relative ether solubility in such a comparison does not give a relative measure of the solubility in membrane lipoids (cf. Collander & Bärlund 1933, p. 90).

Ørskov (1945) has found that the permeability constants of yeast to non-electrolytes are generally 300–1000 times smaller than the corresponding constants of Chara (Collander & Bärlund 1933). It is therefore interesting to note that a base penetrates across the yeast cell membrane on the average only 5–10 times more slowly than a non-electrolyte of the same ether solubility penetrates across the Chara membrane.

Neutral red is the only base whose permeation constant in Chara is known (Collander, Lönegren & Arhimo 1943); it penetrates into Chara a little more rapidly than it would be expected to do into yeast cells (k_{et} for neutral red 4.0, its P constants in Chara $1.6 \cdot 10^{-4}$ cm sec $^{-1}$; k_{et} for ephedrine 5.9, its P in yeast $3.5 \cdot 10^{-5}$ cm sec $^{-1}$).

The yeast cell membrane thus seems to be more permeable to basic compounds than to non-electrolytes. Ørskov's results also show that monovalent amides penetrate into yeast relatively more rapidly than they do into Chara, thus confirming this observation. Whether this fact may be explained as that the lipoids of yeast have a more acid character than those of Chara, is still quite uncertain, because the significance of the pH of the medium is not quantitatively known.

It is also possible roughly to compare the base permeability of Rhoeo (Poijärvi 1928) and yeast. Poijärvi did not use absolute permeability constants, but, knowing the average dimensions of the epidermis cells of Rhoeo and the experimental procedure used by Poijärvi, we can roughly estimate the quotient v/q to be $4.3 \cdot 10^{-3}$ cm. By multiplying the Poijärvi's values, expressed in hour $^{-1}$, by $1.2 \cdot 10^{-6}$ we obtain the approximative absolute constants in cm sec $^{-1}$.

The result of the comparison is shown in Fig. 6. The permeability to bases seems considerably higher in yeast than in Rhoeo. However, according to Collander, Turpeinen and Fabritius (1931), the P values obtained by Poijärvi in Rhoeo are too low. They found in Rhoeo P values for ammonia varying within the limits $2 \cdot 10^{-4}$ – $2 \cdot 10^{-3}$ cm sec $^{-1}$ (recalculated values), thus being of the same order of magnitude or higher than the corresponding values in yeast.

The sieve-effect seems to be of the same order of magnitude in both cases, but the bases having molecules with several polar groups penetrate relatively more rapidly into Rhoeo than into yeast.

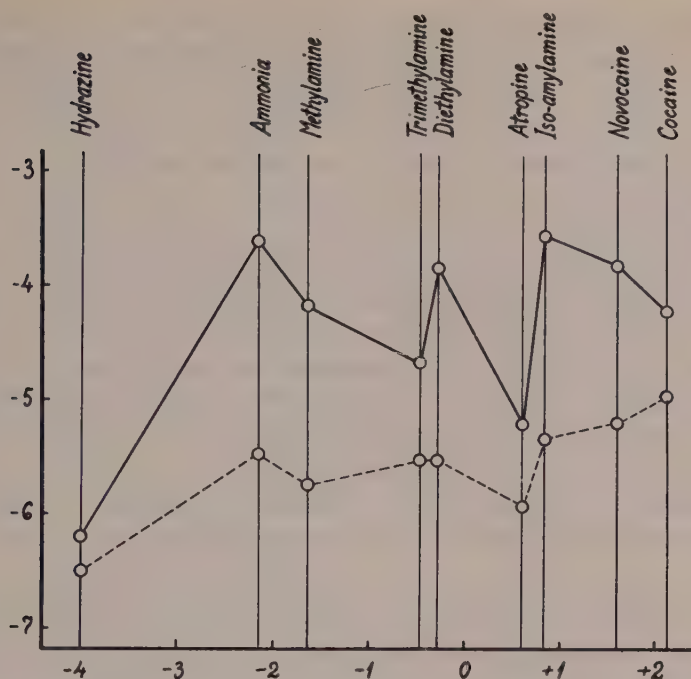


Fig. 6. Comparison between *Rhoeo* and yeast. Abscissa: $\log k_{et}$. Ordinate: $\log P$. The continuous line lies along the points for yeast, the dotted line those for *Rhoeo*.

In general, it may be stated that the permeability properties of yeast are of fairly normal type. In talking about the high permeability of yeast it must be remembered that the high activity of yeast in metabolism processes cannot be attributed to the physical properties of the plasma membrane, i.e. that the rapid adsorption of such substances as fermentable carbohydrates is not a simple diffusion phenomenon but a much more complicated one in which selective systems are involved (cf. Runnström and Sperber 1938).

Summary

The permeability of baker's yeast to 19 different weak bases has been determined. The method is based on the colour change caused by the penetration of base in neutral red stained yeast cells. The time required to reach a certain standard colour is used as a measure of the penetration velocity. In this way it has been possible to determine even very high permeation velocities.

The results show that the lipid solubility is the most important factor. However, the smallest molecules (ammonia, methylamine and hydrazine) penetrate 10^2 — 10^3 times faster than somewhat larger molecules of corresponding lipid solubility. Thus the yeast cell shows a very distinct sieve-effect.

The sieve-effect cannot be explained on the basis of the theory developed by Danielli. It is obvious that molecule size plays a more important part than is suggested by him.

It is probable that the sieve-effect depends on the orientation factor in some such way as that suggested by Wartiovaara. An attempt to apply his hypothesis to alkylamines and ammonia shows that there is a close correlation between the product of the permeation constant and the transverse moment of molecular inertia on the one hand and the relative ether solubility on the other hand. Thus the yeast plasma membrane can be considered as a homogeneous system in a first approximation.

Some attempts to compare the permeability properties of the yeast cell with other cell types indicate that the permeability of the yeast cell is fairly normal.

I wish particularly to thank Prof. R. Collander, who has inspired this investigation and with never-ceasing interest followed my work. I also wish to express my sincere gratitude to the Chief of the Wenner-Gren Institute, Prof. J. Runnström and to Dr. Mignon Malm as well, for the excellent working conditions they arranged for me. For valuable discussions I wish to thank Dr. K. Loimaranta, who has kindly checked the mathematical part of this work, as well as Dr. K. Groth, Mr. M. Hellsten and Dr. V. Wartiovaara.

References

- Äyräpää, T.: The ionization constant and the heat of neutralization of l-ephedrine. — *Svensk Kemisk Tidskrift* 62:135. 1950.
- Bates, R. G.: Definition of pH scales. — *Chem. Rev.* 42:1. 1948.
- Bjerrum, J.: Metal ammine formation in aqueous solution. — Copenhagen 1941.
- Clark, W. M.: The determination of hydrogen ions. — 3d ed., Baltimore 1928.
- Collander, R.: On »lipoid solubility». — *Acta Physiol. Scand.* 13:363. 1947.
- The permeability of plant protoplasts to small molecules. — *Physiol. Plantar.* 2:300. 1949 (a).
- Die Verteilung organischer Verbindungen zwischen Äther und Wasser. — *Acta Chem. Scand.* 3:717. 1949 (b).
- The distribution of organic compounds between iso-butanol and water. — *Acta Chem. Scand.* 4:1085. 1950.
- & Äyräpää, T.: A simple permeability experiment for class work and demonstration purposes. — *Acta Physiol. Scand.* 14:171. 1947.
- & Bärlund, H.: Permeabilitätsstudien an *Chara ceratophylla*. — *Acta Bot. Fenn.* 11:1. 1933.
- , Lönegren, H. & Arhimo, E.: Die Permeationsvermögen eines basischen Farbstoffes mit demjenigen einiger Anelektrolyte verglichen. — *Protoplasma* 37:527. 1943.

- , Turpeinen, O. & Fabritius, E.: Die permeabilität der Rhoeo-Zellen für Ammoniak und Essigsäure. — *Protoplasma* 13: 348. 1931.
- & Wikström, B.: Die Permeabilität pflanzlicher Protoplasten für Harnstoff und Alkylharnstoffe. — *Physiol. Plantar.* 2: 233. 1949.
- Conway, E. J. & O'Malley, E.: Formation of H-ions in high concentrations by ordinary baker's yeast. — *Nature* 153: 652. 1944.
- Davson, H. & Danielli, J. F.: The permeability of natural membranes. — Cambridge 1943.
- Fink, H. & Kühles, R.: Beiträge zur Methylenblaufärbung der Hefezellen und Studien über die Permeabilität der Hefezellmembran. — *Zeitschr. physiol. Chem.* 218: 65. 1933.
- Hall, N. F. & Sprinkle, M. R.: Relations between the structure and strength of certain organic bases in aqueous solution. — *J. Am. Chem. Soc.* 54: 3469. 1932.
- Harned, H. S. & Owen, B. B.: The thermodynamic properties of weak acids and bases in salt solutions. — *J. Am. Chem. Soc.* 52: 5079. 1930.
- Harvey, E. N.: Studies on the permeability of cells. — *J. Exp. Zool.* 10: 507. 1911.
- Jacobs, M. H.: Some aspects of cell permeability to weak electrolytes. — *Cold Spring Harbor Symposia* 8: 30. 1940.
- Jacques, A. G.: The kinetics of penetration. X. Guanidine. — *Proc. Nat. Acad. Sci.* 21: 488. 1935.
- The kinetics of penetration. XVI. The accumulation of ammonia in light and darkness. — *J. Gen. Physiol.* 22: 501. 1939.
- Kolthoff, I. M.: Dissoziationskonstante, Löslichkeitsprodukt und Titrierbarkeit von Alkaloiden. — *Biochem. Zeitschr.* 162: 289. 1925.
- Mahdi Hassan, S.: Die Wasserstoffionenkonzentration im Zellinnern von *Fusarium lini* B. und Hefe. — *Biochem. Zeitschr.* 226: 203. 1930.
- Malm, M.: Über die Permeabilität der Hefezellen und die von den permeierender Stoffen, insbesondere Fluorwasserstoff, bedingten Plasmaveränderungen. — *Arkiv f. kemi, mineral. geol.* 25 A. No 1. 1947.
- Marklund, G.: Vergleichende Permeabilitätsstudien an pflanzlichen Protoplasten. — *Acta Bot. Fenn.* 18: 1. 1936.
- Musfeld, W.: Versuche über die Aufnahme von Zucker durch Hefezellen. — *Ber. Schw. Bot. Ges.* 52: 585. 1942.
- Ørskov, S. L.: Investigations on the permeability of yeast cells. — *Acta Pathol. Microbiol. Scand.* 22: 523. 1945.
- Osterhout, W. J. V.: The adsorption of electrolytes in large plant cells. — *Bot. Rev.* 2: 283. 1936.
- Overton, E.: Über die osmotischen Eigenschaften der Zelle in ihrer Bedeutung für die Toxikologie und Pharmakologie. — *Vierteljahrsschr. Naturf. Ges. Zürich* 41: 383. 1896.
- Pojärvi, L. A. P.: Über die Basenpermeabilität pflanzlicher Protoplasten. — *Acta Bot. Fenn.* 4: 1. 1928.
- Rashevsky, N. & Landahl, H. D.: Permeability of cells, its nature and measurement from the point of view of mathematical biophysics. — *Cold Spring Harbor Symposia* 8: 9. 1940.
- Runnström, J. & Sperber, E.: Zur Kenntnis der Beziehungen zwischen Permeabilität und Stoffwechsel der Hefezelle. — *Biochem. Zeitschr.* 298: 340. 1938.
- Schwarzenbach, G.: Die Dissoziationskonstante von Hydrazin. — *Helv. Chim. Acta* 19: 178. 1936.
- Wartiovaara, V.: The permeability of the plasma membranes of *Nitella* to normal primary alcohols at low and intermediate temperatures. — *Physiol. Plantar.* 2: 184. 1949.
- Zur Erklärung der Ultrafilterwirkung der Plasmahaut. — *Physiol. Plantar.* 3: 462. 1950.

Inorganic Carbon Sources of Green Algae. II. Carbonic Anhydrase in *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*

By

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It has been shown previously (Österlind 1950) that *Scenedesmus quadricauda* and *Chlorella pyrenoidosa* differ in their ability to use bicarbonate for growth. *Scenedesmus* uses it very well, whereas *Chlorella* cannot use it at all. In free CO_2 the two algae grow at about the same rate. Steemann Nielsen and Kristiansen (1949) have examined *Elodea canadensis* and *Fontinalis dalecarlica* with respect to the presence of carbonic anhydrase. These two species differ in about the same way as *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*, although *Scenedesmus* seems to use HCO_3^- better than *Elodea* in relation to the usage of free CO_2 . However, they found no difference in the enzyme content between *Elodea* and *Fontinalis*.

It was considered that it might be of interest, also to examine the two different algae with respect to the content of carbonic anhydrase. The results are reported in the present paper.

The experiments were performed in a similar way as those of Steemann Nielsen and Kristiansen (1949), but the sensitivity was increased by using a larger volume of the suspension to be examined. The algae from 1 litre of culture solution (nutrient solution with carbonate buffer, aerated with about 2 % of CO_2 in air) were centrifuged and washed once in a mixture of equal volumes of 0.02 M l(+)-cysteine hydrochloride and 0.4 M phosphate buffer (pH 6.8). This mixture could not be stored, because a white precipitate was formed after some days. The cells (volume about 1 ml) were transferred to a small mortar and enough Pyrex glass powder was added to form a thick paste, which was ground for about 15 minutes. When it had been determined microscopically that most of the cells had broken, the paste was transferred to a centrifuge tube by means of about 2 ml of the cysteine —

phosphate mixture and the glass powder was settled by centrifuging for a short time. The rest of the suspension was used directly for the enzyme determinations.

In a 1" lucite rod a central $\frac{3}{4}$ " bore was turned. By means of an edge-formed piece of a $\frac{3}{4}$ " rod, the bottom of the bore was divided into two parts. On one side of the edge, 0.5 ml of the algal suspension in the cysteine — phosphate mixture was placed; on the other side, 0.5 ml of 0.2 M NaHCO_3 , dissolved in 0.02 M NaOH. The vessel was closed with a rubber stopper and connected with a Warburg manometer by means of fine pressure tubing. The vessel was placed in a water bath at 15° C and after having reached temperature equilibrium, it was shaken rapidly (380 complete oscillations per minute). The manometer was read every 10 seconds after the beginning of the shaking.

A comparison should not be made against a control, where no algal suspension has been added to the cysteine — phosphate mixture. The rate of gas evolution depends on the concentration of the phosphate mixture, and this is not constant from case to case, but depends on the amount of cells per unit of phosphate mixture added, on the evaporation during grinding in the mortar, and on the acidity of the cellular content. Therefore, part of the green suspension, heated in a water bath to 90° C for 5 minutes, was used as a comparison. The evaporation during this heating has been shown to have no influence and if it had, would operate in the opposite direction to the inactivation of the enzyme.

Each curve in the figures represents the mean value of two experiments with the same suspension. The two experiments did not differ more than 1 or 2 mm.

From Figs. 1 and 2 it is seen that both algae contain carbonic anhydrase. The activity of *Scenedesmus* seems to be greater than that of *Chlorella*, but this may be an accident. The activity may depend on the treatment of the cells during the preparation of the suspension. Another experiment with *Scenedesmus quadricauda* showed about the same activity as that reported in Fig. 2 for *Chlorella pyrenoidosa*, although the chlorophyll content in the two suspensions did not differ very much. Another experiment with *Chlorella* showed a slightly lower, though still distinct, activity than that reported in Fig. 2.

Control experiments have also been made with *Elodea canadensis*, *Sambucus nigra* and *Fagus orientalis* from the Botanical garden of Uppsala. The experiments were made in September 1950. The activity of *Elodea* was a little higher than that of *Chlorella* in Fig. 2, but the chlorophyll content of the *Elodea* suspension was 6 times less than that of *Chlorella* (density 1.2 resp. 7.2, as described below). A rather dilute suspension from *Sambucus nigra* (density 0.6) had a very high activity. Although only 0.2 ml of suspension was used, in accordance with the method employed by Steemann Nielsen and Kristiansen (1949), 25 cm on the manometer scale was reached after only 15 seconds, the curve after heating being the same as in Fig. 2. An experiment with *Fagus orientalis* (0.2 ml suspension, chlorophyll content 0.4) showed no activity at all.

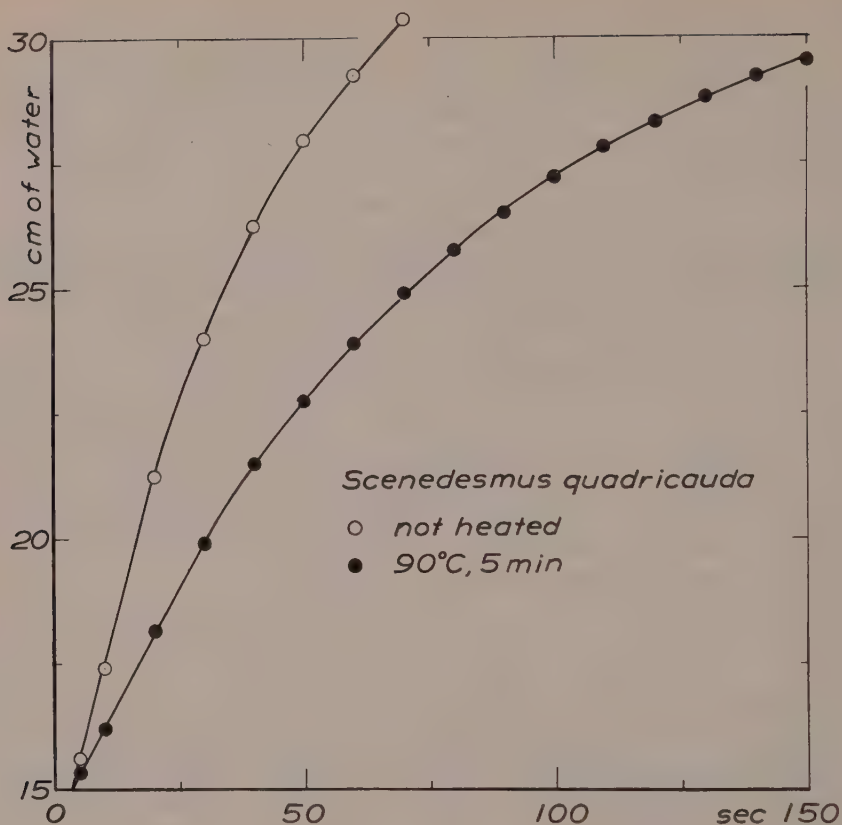


Fig. 1. Rate of output of CO_2 when mixing 0.5 ml suspension of *Scenedesmus quadricauda* in phosphate buffer with 0.5 ml bicarbonate solution.

The chlorophyll content was determined by boiling 1 ml of the suspension with 5 ml of methanol for 1 minute, suction-filtering the solution and diluting it to 10 ml (cf. Rodhe 1948, p. 12). If necessary, a further dilution was made or less than 1 ml of the suspension was used. The amount of chlorophyll is given as the density of the solution in 1 cm absorption cells at the maximum at 6660 Å in a Beckman spectrophotometer, corrected for a possible further dilution.

It is seen from the experiments with the algae that both of them contain carbonic anhydrase, although the amount seems to be rather small. In this case, just as in that of *Elodea* and *Fontinalis*, it therefore seems improbable that the difference in bicarbonate assimilation has any connection with the content of carbonic anhydrase.

If the enzyme is located at suitable parts of the cellular structure and its concentration is high enough to enhance the hydration of CO_2 or dehydration

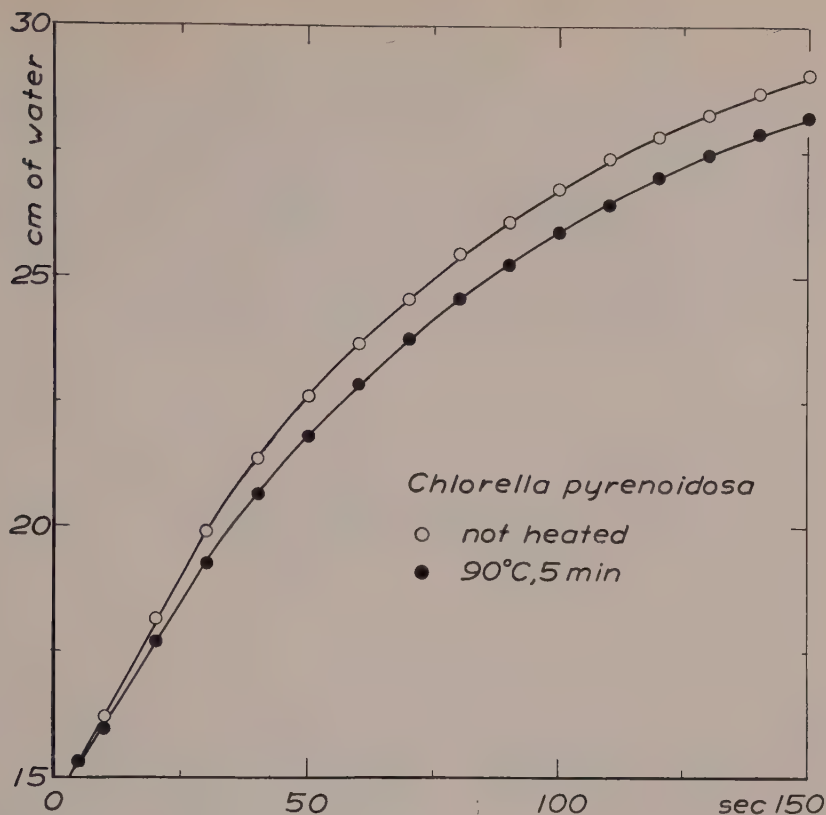


Fig. 2. Rate of output of CO₂ when mixing 0.5 ml suspension of *Chlorella pyrenoidosa* in phosphate buffer with 0.5 ml bicarbonate solution.

of HCO_3^- sufficiently, so that these reactions are not limiting, then the differences with respect to the bicarbonate assimilation must be looked for prior to the carboxylation processes. It therefore seems most probable that the differences are caused by differences in the transport mechanism through the protoplasmic membrane.

Summary

The difference with respect to the bicarbonate assimilation between *Scenedesmus quadricauda* and *Chlorella pyrenoidosa* may not be ascribed to differences in the content of carbonic anhydrase. Most likely it is due to differences in the protoplasmic membrane.

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References

- Österlind, S.: Inorganic carbon sources of green algae. I. Growth experiments with *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. — *Physiol. Plant.*, 3: 353. 1950.
- Rodhe, W.: Environmental requirements of fresh-water plankton algae. Experimental studies in the ecology of phytoplankton. — *Symbol. Botan. Upsaliens.*, 10 (1): 1. 1948.
- Steemann Nielsen, E. & Kristiansen, J.: Carbonic anhydrase in submersed autotrophic plants. — *Physiol. Plant.*, 2: 325. 1949.

The Effects of Cytochrome Oxidase Inhibitors on the Cytochrome Oxidase and Respiration of the Fungus *Myrothecium verrucaria*

By

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Introduction

This study of the terminal oxidase of the mycelium of the cellulolytic fungus *Myrothecium verrucaria* has shown that although the respiration of the intact organism is not inhibited by high partial pressures of carbon monoxide and inappreciably inhibited by 10^{-3} M hydrogen cyanide, the organism contains a typical cytochrome oxidase. This oxidase has been demonstrated in mycelial extracts by spectrophotometric and manometric techniques, and cytochrome *c* has been observed in the intact organism. The oxidase exhibits the typical sensitivity to the well known cytochrome oxidase inhibitors as well as high activity at low partial pressures of oxygen. It is associated with particulate matter, since it is almost completely sedimented at centrifugal fields of 25,000 g. for one hour. Though the respiration of the mycelium is relatively insensitive to hydrogen cyanide, phenylthiourea, and substances forming copper complexes, it is strongly inhibited by hydrazoic acid. We have been unable to demonstrate the presence of polyphenol oxidase.

Materials and Methods

Preparation of the mycelium and mycelial extracts. — The technique of handling this filamentous fungus in respirometry has been described by Darby and Goddard (2). Briefly, it consists of propagating the mycelium in

a medium containing malt extract, 50 g., glucose, 50 g., NH_4NO_3 , 3 g., K_2HPO_4 , 2.1 g., KH_2PO_4 , 2.9 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.2 g., and distilled H_2O , to 1000 ml., pH *ca.* 6.5. The mycelial pellets formed in 125 ml. shaker flasks in two days at 30° C are harvested, washed in buffer (0.0167 M K-phosphate + 0.001 M MgSO_4 , pH 6.8 to 7.2) and suspended finally in buffer, usually with 0.02 M glucose added. This suspension, appropriately diluted, is used directly in the Warburg flasks at 30° C or is used to prepare mycelial extracts.

Active oxidase preparations were obtained by grinding the mycelium in the cold with finely powdered glass (100 μ or less) or levigated alumina (10 μ or less) in a chilled mortar or Potter-Elvehjem mill. The macerate was taken up in buffer (pH 7.2) and centrifuged at slow speed in the cold to remove the grinding material and mycelial remnants. The cytochrome *c* was obtained from Wyeth Corporation in ampoules at 50 mg./5 ml. This was diluted as required with pH 7.1 or 7.2 buffer. Reduction for spectrophotometric analysis was accomplished immediately prior to use by the addition of a few small crystals of sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) and the excess reductant was removed by aeration for about five minutes. The spectrophotometric determinations were made on a Beckman model DU quartz spectrophotometer at 550 $m\mu$ with corex cells of 1.00 cm light path. The oxidase was added to the reduced cytochrome *c* at zero time by means of a hypodermic syringe.

The manometric experiments were by the usual Warburg techniques with inhibitors dissolved in buffer added from the side arm after a preliminary period to establish the control rate. Control flasks with buffer tipped in from the side arm were also run. Cytochrome oxidase was assayed by the increase in O_2 uptake upon the addition of cytochrome *c* over the rates obtained without added cytochrome *c*. Hydroquinone was used as electron donor. The manometric experiments were at 30° C, and the spectrophotometric ones at about 25° C, and the concentrations given are the final concentrations in the vessel.

In cyanide experiments the KCN in buffer was added directly to the flask or cell. Krebs (11) KOH-KCN mixtures were used in the respiration experiments with cyanide.

Carbon monoxide was obtained either by decomposition of formic acid or from tank CO (Matheson). The latter contained about one per cent each of N_2 , H_2 and saturated hydrocarbons. Carbon dioxide and other acidic substances were removed by passing the CO through alkali. Gas mixtures were prepared by mixing the purified CO or tank N_2 with tank O_2 at the ratio of 95/5 or 90/10. Respirometer atmospheres were altered either by flushing the gas mixtures through at the rate of 2 liters in 20 minutes or by the evacuation technique (15) while the vessels were shaking in the bath.

Experimental

Since cytochrome *c* is but slowly oxidized by atmospheric oxygen, the presence of cytochrome oxidase in the extracts could be demonstrated by its catalysis of the oxidation of ferro cytochrome *c* by dissolved oxygen. The oxidation was followed on the spectrophotometer at 550 m μ , since the lower light absorption of the ferri cytochrome *c* means a decrease in optical density as the oxidation proceeds. Concentrations of cytochrome *c* were calculated from the relation (15):

$$C = \frac{D}{\epsilon l} \quad (1)$$

where C = moles per ml.

$D = \log I_0/I_x$ I_0 = incident intensity

I_x = transmitted intensity

$\epsilon_{\text{red.}} = 2.81 \times 10^7$ moles⁻¹ cm at 550 m μ

$\epsilon_{\text{ox.}} = 0.955 \times 10^7$ moles⁻¹ cm at 550 m μ

l = light path in cm.

The relation between concentration of ferri cytochrome *c* and the optical density is:

$$C_{\text{ox}} = \frac{D - \epsilon_{\text{red.}} C_T}{l (\epsilon_{\text{ox.}} - \epsilon_{\text{red.}})} \quad (2)$$

where C_{ox} = conc. in moles per ml. of ferri cytochrome *c*

C_T = total concentration of cytochrome

and other terms as above.

A typical experiment is shown in fig. 1. The optical density of the oxidase cell changed from 0.787 to 0.662, a difference of 0.125 in the first minute while the buffer cell changed from 0.815 to 0.805 in the first 7 minutes, a difference of 0.007 per minute. Thus, in the first minute 24 per cent of the ferro cytochrome *c* was oxidized in the presence of the oxidase while 1.2 per cent was oxidized in the control. It is difficult to calculate precisely the completeness of oxidation or the percentage inhibition by the control, as the turbidity of the oxidase and the rapidity of the oxidation makes difficult the exact determination of the change in D . However, from 1/5 diluted oxidase, taking the initial D . as 0.93 and final value as 0.38, the calculation indicates 97 per cent of the cytochrome *c* has been oxidized. Diluting the oxidase 1/5 results in a proportional decrease in rate.

Cytochrome oxidase was also demonstrated with Warburg respirometers by the increased rate of oxidation of hydroquinone upon addition of cyto-

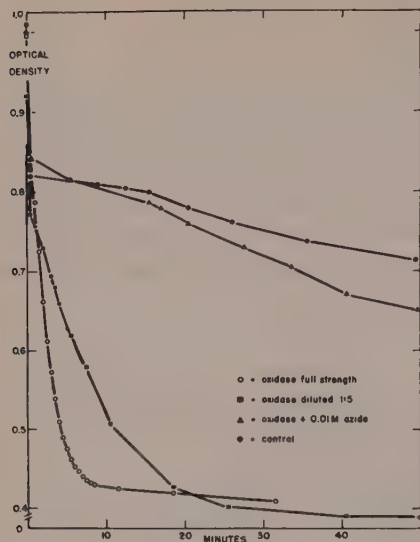


Fig. 1. Spectrophotometric demonstration of cytochrome oxidase in mycelial extract. Each cell contained 2.5 ml. of ferro cytochrome *c* in pH 7.1 buffer, plus 0.5 ml. oxidase, or of oxidase+ NaN_3 , or buffer. Cytochrome *c* concentration in cell = 3.0×10^{-5} M.

chrome *c*, as is shown in fig. 2. The rate is decreased, but not proportionately, upon dilution of the oxidase 1 to 2.

Effect of inhibitors on cytochrome oxidase. — Though the mycelium respiration is not inhibited by CO and not appreciably by 10^{-3} M HCN, the cytochrome oxidase in the extracts is inhibited strongly by HCN, CO, and HN_3 . The azide inhibition is shown in fig. 1 and is estimated to be about 92 per cent during the first three minutes. However, the inhibition may be more complete than this, as Horecker and Stannard (8) have shown that NaN_3 increases the rate of autoxidation. The effect of cyanide in a manometric experiment is shown in fig. 3 while fig. 4 shows its action in a spectrophotometric

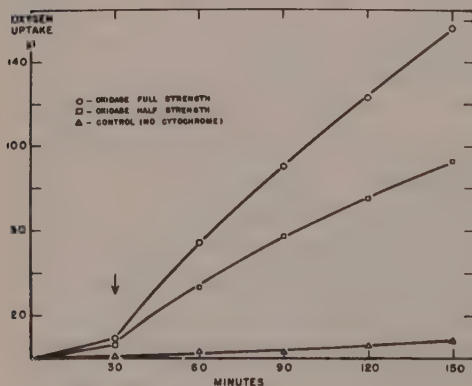
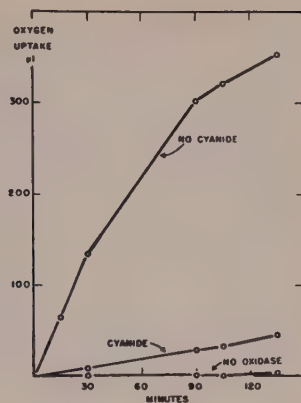


Fig. 2. Oxidation of hydroquinone by mycelial extract in the presence of cytochrome *c*. Vessels contained 1.9 ml. of oxidase, cytochrome *c* at 1.75×10^{-6} M, and hydroquinone at 0.013 M; total volume 2.3 ml. All solutions in phosphate buffer pH 7.2, 0.05 M; temp. 30°C ; cytochrome *c* added from side arm at arrow.

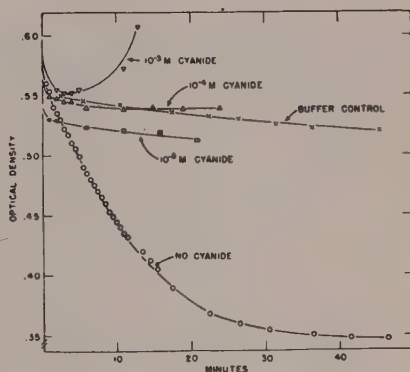
Fig. 3. The effect of cyanide at 1.8×10^{-3} M on the inhibition of the cytochrome oxidase of mycelial extract; hydroquinone at 0.013 M, cytochrome *c* at 7×10^{-6} M; phosphate buffer pH 7.2; temp. 30° C.



metric experiment. Complete inhibition is obtained at 10^{-5} M, which might be expected from Stannard and Horecker's (14) determination of the heart cytochrome oxidase cyanide dissociation constant 5×10^{-7} M. The slight increase in optical density at 10^{-4} M HCN and the marked increase at 10^{-3} M HCN can hardly be due to the formation of the ferri cytochrome *c*-cyanide compound (7), as the increased absorption due to the cyanide complex is inadequate to explain the observed rise in optical density. It is possible that the cyanide is combining with some non-cytochrome material that absorbs light at $550 \text{ m}\mu$ more strongly than the cytochrome-cyanide complex or the cytochrome added to the cells was not completely reduced, and reducing systems added with oxidase are slowly reducing the ferri cytochrome *c* present.

The carbon monoxide inhibition of cytochrome oxidase is demonstrated in fig. 5. The inhibition, calculated from the slopes of the curves, is about 83 per cent, ignoring the differences in O_2 tension. This is based on the

Fig. 4. The inhibition of cytochrome oxidase demonstrated spectrophotometrically. The cells contained 2.0 ml. of reduced cytochrome *c*, 0.5 ml. of oxidase diluted 1:10, and 0.5 ml. of cyanide or buffer. All solutions made up in buffer. Final cytochrome *c* concentration in cells 2×10^{-5} M.



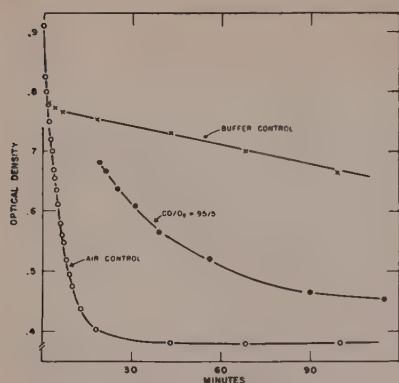


Fig. 5. The effect of CO on the oxidase in the dark. All cells contained 2.5 ml. of ferro cytochrome *c* and 0.5 ml. of buffer or of oxidase. Final cytochrome *c* concentration 2.8×10^{-5} M. CO/O₂ mixtures were bubbled through the cytochrome *c* and oxidase in the dark for approximately 20' before mixing. The CO/O₂ cell was kept in the dark until the first readings, and then was exposed to the weak light of the spectrophotometer at 550 mμ.

finding (2) that in the manometer the rate with 10 per cent O₂ was 80 per cent of that with air, moreover, an excess of O₂ is present here, as the dissolved O₂ in equilibrium with 5 per cent O₂ in the gas phase is 6.3×10^{-5} M, and only $\frac{1}{4}$ mole of O₂ is used for each mole of cytochrome. The cells were adapted for flushing with CO/O₂ mixture in the dark; and both oxidase and cytochrome *c* were saturated with gas before mixing. The first reading of the CO cell was made after 20 minutes in the dark. Since the action spectrum of relief of CO inhibition is near a minimum at 550 mμ (16), it is thought that little light reversal will result from the low intensity light used in the measurements. Similar experiments conducted with CO/O₂ without excluding laboratory light resulted in no CO inhibition.

Carbon monoxide inhibition of the oxidase in the dark was also obtained in the respirometer with typical light reversal. The flasks were darkened by black Tygon paint or black cloth. Light for reversal was obtained from 6 V lamps immersed in the bath about 2.5 cm below the flasks providing in

Table 1. Effect of carbon monoxide (in CO/O₂ mixtures) on the oxidation of hydroquinone (0.013 M) by mycelial extracts in the presence of cytochrome *c* (7×10^{-6} M). Results are expressed as per cent of N₂/O₂ control for 20–25 minute periods in the dark followed by similar periods in the light.

Experiment	% of control		% reversal
	Dark	Light	
1	35	56	21
2	58	79	21
3	30	50	20
4	57	89	32
5	59	74	15

Table 2. *Effect of centrifugation on the oxidase.* Respirometer test with 0.3 ml hydroquinone (0.013 M), 0.5 ml cytochrome *c* (7×10^{-6} M), 1.0 ml buffer pH 7.2, 0.5 ml oxidase, temp. 30° C. O₂ uptake in μ l./hr.

Fraction	Observed	Corrected for autoxidation	% of control
Crude oxidase (control)	96	75	(100)
Boiled oxidase	17	0	0
Buffer	21	0	0
Supernatant — 700 g., 10' ...	79	58	77
Sediment — 700 g., 10'	38	17	23
Supernatant — 25,000 g., 60' ...	26	5	6.7
Sediment — 25,000 g., 60' ...	87	66	88

intensity at the vessel of 600—700 foot candles. The results of several experiments are summarized in table 1. While it was not always possible to show clear-cut inhibition by CO, when inhibition did occur it was about 30 to 60 per cent under the most favorable conditions.

Other properties of the oxidase. — The oxidase is typical in being associated with particulate matter in the extracts. This was demonstrated by its removal from suspension by high speed centrifugation. When subjected to a centrifugal field of about 25,000 g (in the cold) for about one hour, 88 per cent of the activity was recovered in the centrifugate. At low speed centrifugation most of the activity remained in the opalescent supernatant (table 2).

The oxidase was found to function at low partial pressures of oxygen with high activity. At one per cent oxygen, the oxygen uptake in a typical hydroquinone-cytochrome-oxidase system was 43 μ l in 50 minutes as compared to 57 μ l in air, representing about 75 per cent activity.

In vivo experiments

Effect of cytochrome oxidase inhibitors on respiration. — In experiments with intact mycelium, the respiration was highly resistant to carbon monoxide and relatively so to cyanide although sodium azide caused marked inhibition when measured at suitable pH values. The azide results plotted in figure 6 show that 1×10^{-3} M NaN₃ caused inhibitions of 83, 78, and 18 per cent respectively at pH's of 4.20, 5.13, and 6.20. If we take the dissociation constant of HN₃ as 1.9×10^{-5} M, the concentrations of undissociated HN₃ are as follows:

pH	HN ₃
4.20	7.68×10^{-4} M
5.13	2.80×10^{-4} M
6.20	0.026×10^{-4} M

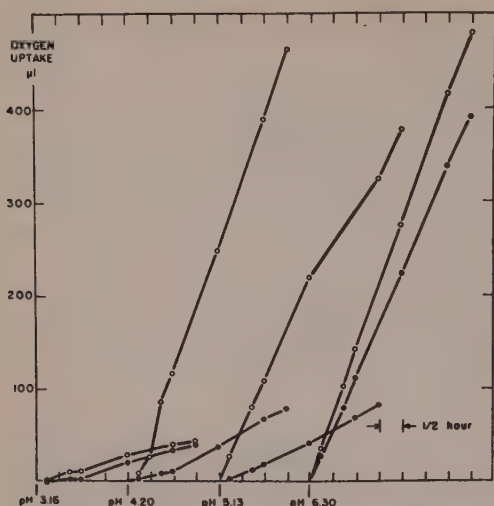


Fig. 6. *Effect of pH on azide inhibition of respiration.* The ordinate is displaced 2 hours to the right for each successive pH. All flasks were started concurrently. Open circles, without azide; close circles, with 0.001 M azide. Temperature = 30° C.

It is apparent that the respiration is inhibited by HN_3 and not N_3^- , agreeing qualitatively with Keilin (10) who found that yeast was not inhibited by 1×10^{-3} azide at pH 7.5 but was 97 per cent inhibited at pH 6.2. Stannard and Horecker (14) showed that extracted heart cytochrome oxidase was inhibited by HN_3 but not N_3^- . At pH 3.16 the azide effect is masked by the inhibition of the respiration by the acidity (2). The respiration and extracted oxidase respond similarly to azide, except that the former requires a higher concentration for equal inhibition.

The sodium azide results lead us to expect that the respiration would be

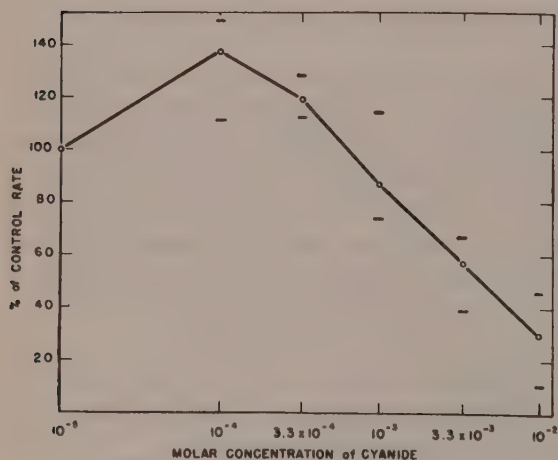


Fig. 7. *Effect of cyanide on the respiration of mycelial pellets.* Temperature = 30° C.

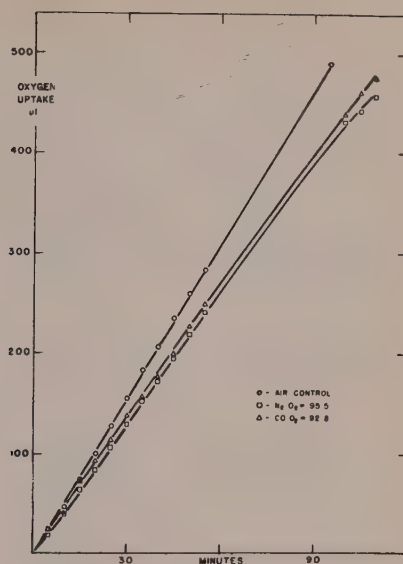


Fig. 8. Effect of carbon monoxide on respiration of the mycelium of *Myrothecium verrucaria* in the dark. Temperature = 30° C.

sensitive to cyanide and as the first experiments at 10^{-3} M gave little or no inhibition, a wide range of concentrations was tested. The results are plotted in figure 7, where each point except at 10^{-5} M represents the mean of 3—4 experiments, and in each experiment duplicate or triplicate vessels were used for each concentration. The results are somewhat variable, and the range is represented in figure 7 by the horizontal bars. This relative insensitivity to cyanide in an organism with a Q_{O_2} of 60 in the presence of 0.02 M glucose was surprising. Similar results were obtained in an experiment without glucose. It is obvious that cyanide is having at least two effects, a stimulation and an inhibition, and it is not possible to calculate the individual effects.

Carbon monoxide at 90 to 95 per cent concentration in mixtures with O_2 failed to inhibit respiration significantly in darkened vessels. A typical experiment is illustrated in figure 8. The CO/O_2 and N_2/O_2 mixtures were flushed serially through duplicate flasks while shaking in the bath at about 2 liters in 20 minutes. There is essentially no difference in rate between the CO flasks and the N_2 controls.

Other oxidases. — Attempts to demonstrate other oxidases have failed. Without direct evidence of the presence of these oxidases, such as is presented in this paper for cytochrome oxidase, inhibitor studies can only be considered suggestive. However, the presence of polyphenol oxidase is negated by the failure of copper inhibitors such as 8-hydroxyquinoline and sodium diethyl-dithiocarbamate to inhibit the respiration (table 3). The absence of inhibition

Table 3. *Effect of enzyme inhibitors on mycelial respiration. Temp.=30° C, pH=6.8.*

Inhibitor	Molarity	% of control rate		Duration hours
		Initial	Final	
8-Hydroxyquinoline	0.0001	98	94	3
	0.001	76	87	
	(Saturated)	59	7	
Sodium diethyl dithiocarbamate	0.0001	99	112	3
	0.001	95	108	
	0.01	96	95	
	0.1	84	82	
o-Nitrophenol	0.0001	91	86	2.5
	0.001	76	83	
	0.01	70	57	
p-Nitrophenol	0.0001	106	99	2.5
	0.001	113	96	
	0.01	34	23	
Phenylthiourea	0.0001	105	107	1.5
	0.001	110	111	
	(Saturated)	98	101	

by phenylthiourea (3) even in saturated solutions is particularly convincing evidence of the non-functioning of polyphenol oxidase in this organism. Moreover, this enzyme could not be demonstrated in extracts when tested in the respirometer with catechol in catalytic quantities (0.1 mg) and hydroquinone as hydrogen donor (3 mg/vessel). This test gives high activities with extracts containing polyphenol oxidase (4). The ineffectiveness of the inhibitors in table 3 strongly suggests the absence of copper containing enzymes as respiratory enzymes in this fungus.

Discussion

The correlation of the occurrence of cytochrome oxidase in organisms whose respiration is inhibited by cyanide, azide, and photoreversibly by carbon monoxide is so high that it is assumed that in the absence of these inhibitions, the cytochrome oxidase is absent. Extracted cytochrome oxidase saturated with ferro cytochrome *c* is highly sensitive to HCN and HN₃ with 50 % inhibition at 5 and 7×10^{-7} M respectively (14). Cyanide resistant respiration is also well known (1, 13) and cyanide stimulation of respiration has been well recognized since the studies of Hanes and Barker (6).

If a conclusion on the occurrence of cytochrome oxidase for this fungus were based on respiratory studies alone, the decision would certainly have

been that cytochrome oxidase was absent; yet the extracts show clearly the presence of the enzyme.

Cytochrome *c* was observed in a heavy suspension of mycelium from young shake cultures. A qualitative judgment was that the cytochrome *c* concentration was low for an organism of this high Q_{O_2} . A faint band was observed suggesting the presence of cytochrome *a*. These observations were made possible through the generosity of Dr. Robin Hill of the University of Cambridge who placed his instruments and experience at the disposal of one of the authors.

The apparent anomaly between the cyanide and carbon monoxide results in inhibiting the oxidase in extracts but not the respiration of the intact organism may be due to one of several reasons:

1. The cytochrome oxidase in the organism is not saturated with ferro cytochrome *c* in spite of the high Q_{O_2} (in this case of 60). This is the case in dormant *Neurospora* ascospores (5) which have a low Q_{O_2} (0.025) and are insensitive to cyanide, while the respiration of the activated or germinating spores ($Q_{O_2}=10$) is sensitive to CO and HCN. If this is the explanation, then there is relatively an excess of cytochrome oxidase in relation to cytochrome *c*.

2. Impermeability of the cells to CO or HCN seems highly improbable, and particularly so since HCN causes a respiratory stimulation.

3. That the enzyme in the cell is protected from the inhibitor, either by the orientation of the enzyme within a structural unit, or by the presence of a competing reaction removing the inhibitor as fast as it enters the cell. Though the former may be true, no experimental approach to test it is evident. Though HCN combines with disulfides and ketones, it seems improbable that these reactions would maintain a low enough concentration to prevent enzyme inhibition, if the intracellular oxidase is as sensitive as the extracted enzyme.

4. Though cytochrome oxidase is present in the fungus, there is another terminal oxidase which is adequate to account for the observed respiration. This does not mean that cytochrome oxidase is functionless in the intact organism, but either a small percentage of the total electron transfer occurs through it, or the alternate oxidase functions during its inactivity. Laties (12) has found a cyanide resistant oxidase in chloroplasts, and James and Beevers (9) have shown that the respiration of the spadix of *Arum* is carried over a flavoenzyme system, insensitive to cyanide.

The explanation of the observed results can come only from further experimental attack. However, one should be cautious in concluding that cytochrome oxidase is absent because the respiration is essentially insensitive to cyanide and carbon monoxide.

Summary

Extracts of the fungus, *Myrothecium verrucaria*, contain an active cytochrome oxidase which is typical in its sensitivity to low concentrations of hydrazoic acid and hydrogen cyanide and its photoreversible sensitivity to high partial pressures of carbon monoxide. However, the respiration of the intact mycelium is not inhibited by 95 % carbon monoxide, nor appreciably by 10^{-3} M cyanide; lower cyanide concentrations bring about a respiratory stimulation. Hydrazoic acid inhibits the respiration. No polyphenol oxidase could be demonstrated.

References

1. Commoner, B.: Cyanide inhibition as a means of elucidating the mechanisms of cellular respiration. — *Biol. Rev. Cambridge Phil. Soc.* 15: 168. 1940.
2. Darby, R. T. & Goddard, D. R.: Studies of the respiration of the mycelium of the fungus *Myrothecium verrucaria*. — *Amer. J. Bot.* 37: 379. 1950.
3. Dubois, K. P. & Erway, W. P.: Studies on the mechanism of action of thiourea and related compounds. — II Inhibition of oxidative enzymes and oxidations catalyzed by copper. — *J. Biol. Chem.* 165: 711. 1946.
4. Goddard, D. R. & Holden, C.: Cytochrome oxidase in the potato tuber. — *Arch. Biochem.* 27: 41. 1950.
5. Goddard, D. R. & Smith, P. K.: Respiratory block in the dormant spores of *Neurospora tetrasperma*. — *Plant Physiol.* 13: 241. 1938.
6. Hanes, C. S. & Barker, J.: The physiological effect of cyanide. — I. The effects of cyanide on the respiration and sugar content of the potato at 15°C. — *Proc. Roy. Soc. London B* 108: 95. 1931.
7. Horecker, B. L. & Kornberg, A.: The cytochrome c-cyanide complex. — *J. Biol. Chem.* 165: 11. 1946.
8. Horecker, B. L. & Stannard, J. N.: The cytochrome c-azide complex. — *J. Biol. Chem.* 172: 589. 1948.
9. James, W. O. & Beevers, H.: *New Phytologist* (in press). 1950.
10. Keilin, D.: The action of sodium azide on cellular respiration and on some catalytic oxidation reactions. — *Proc. Roy. Soc. London B* 121: 165. 1937.
11. Krebs, H. A.: Metabolism of amino acids. — III. Deamination of amino acids. — *Biochem. J.* 29: 1620. 1935.
12. Laties, G.: An oxidative, cyanide-insensitive enzyme system in the chloroplasts of a higher plant. — *Arch. Biochem.* 27: 404. 1950.
13. Marsh, P. B. & Goddard, D. R.: Respiration and fermentation in the carrot, *Daucus Carota*. II. Fermentation and the Pasteur effect. — *Amer. J. Bot.* 26: 724. 1939.
14. Stannard, J. N. & Horecker, B. L.: The in vitro inhibition of cytochrome oxidase by azide and cyanide. — *J. Biol. Chem.* 172: 599. 1948.
15. Umbreit, W. W., Burris, R. H. & Stauffer, J. E.: *Manometric techniques and tissue metabolism*. — Burris Pub. Co. Minneapolis 1949.
16. Warburg, O.: Heavy metal prosthetic groups. Transl. by A. Lawson. — Oxford Univ. Press 1949. p. 144.

On Auxin Antagonists and Synergists in Root Growth

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Introduction

It is generally assumed that the auxin concentration necessary for optimum root growth is extremely low (about 10^{-10} M), and it is possible that the normal concentration of intact roots is slightly supra-optimal (Thimann 1948 p. 46). Most reports on positive auxin effects on root growth also refer to isolated or decapitated roots, or to stimulations occurring after the external auxin application has been removed. In any case the auxin concentrations necessary to cause a conspicuous inhibition are so low, that many chemically related substances can be applied at much higher doses without being definitely toxic. Auxin-inhibited roots could thus be expected to be valuable test objects in the search for competitive auxin antagonists, especially as such antagonists should cause positive growth effects which are probably much more specific than the growth inhibitions obtained for organs with optimal or sub-optimal auxin content.

The first experiments were designed to show the effect of 1-naphthol (Nf) upon the activity of 1-naphthaleneacetic acid (NAA). If the naphthalene nucleus is responsible for the attachment of the NAA-molecules or ions to the proper places in the cytoplasmic framework, it should perhaps be possible to displace these molecules by another, inactive naphthalene derivative. The data obtained in the first experiments did really indicate that 1-naphthol gave such effects. Among some other naphthalene derivatives which were placed at our disposal by Prof. A. Fredga, the α -(1-naphthyl-methylsulfide)-propionic acid [$C_{10}H_7 \cdot CH_2 \cdot S \cdot CH(CH_3) \cdot COOH$] (NMSP) turned out, however, to be even less harmful to the roots than 1-naphthol and to give much stronger antagonistic effects when applied together with

NAA or other growth substances (2,4-D or 2,4-dichlorophenoxyacetic acid; IAA or indole-3-acetic acid). Consequently NMSP was used for most of the experiments reported here. For the sake of comparison an auxin synergist, triiodobenzoic acid (TIBA), was also tested with the same methods.

Methods

The inhibition of root growth by various growth substances has often been used for the determination of their concentration or activity. In recent applications the seedlings have usually been grown in Petri dishes on filter paper moistened with the test solutions (Swanson 1946: corn, Ready & Grant 1947: cucumber, Moewus 1949 b: *Lepidium sativum*). In this study flax (*Linum usitatissimum*), var. Concurrent B, was used as a test plant, and the seedlings were at first grown on moist filter paper as usually done:

F-test. The seeds were allowed to swell for about 2 hours in tap water and were then transferred to moist filter paper in large Petri dishes (20 cm diam.). The covered dishes were placed in a dark temperature control cabinet at 21° C. In order to obtain straight and regular root growth the dishes were tilted 60° out of the horizontal. After 24 hours seedlings with roots of 3 mm length were selected and transferred to the test dishes which were placed in the same position and in the same control cabinet. To each dish (12 cm diam.) was added 10 ml solution and 20 seedlings were placed on the moistened filter paper. After about 19 hours the roots were then measured again. All tested substances were dissolved in a phosphate buffer of pH ca. 5.9 (1 Na₂HPO₄ + 9 KH₂PO₄; total concentration 6.7 mM) which was also used for the control seedlings. The stock solutions of the growth substances were, if necessary, neutralized with NaOH.

It soon turned out, however, that the roots sometimes showed a tendency to curve away from the filter paper. For solutions containing NMSP this phenomenon was especially strong and regularly occurring. As the uptake of the active substances into the roots could evidently be influenced by these curvatures, it seemed appropriate to repeat the experiments under ordinary solution culture conditions:

S-test. The seedlings were reared as described for the *F*-tests, but at 25° C. At 7 mm root length they were transferred to paraffined cork plates, each with 14 perforations for the seedlings. These cork holders were floated on the test solutions, each holder being placed in a glass tube of 4 cm diameter, containing 75 ml solution. After a growth period of 17 hours at 25° C the roots were measured again. As the hypocotyl will begin to grow during this period (2–3 mm) it was found advisable to mark the roots with Indian ink at a distance of 5 mm from the tip, thus obtaining an easily detectable reference point for the measurements. The solutions contained the same phosphate buffer as in the *F*-tests, but 5 mM Ca(NO₃)₂ was also added to obtain good growth.

Experimental Results

The results obtained for pure solutions of the different substances tested are summarized in figures 1 and 2. In order to facilitate a comparison the molar concentrations needed for 50 % inhibition are also presented (table 1). The two test methods have apparently given results of quite the same type, the *S*-test, however, being about 20 times more sensitive than the *F*-test. For seedlings of *Lepidium sativum*, grown on filter paper moistened with unbuffered solutions, Moewus (1949 b) found 2.4×10^{-7} M IAA necessary for 50 % inhibition of the root growth, while Audus (1949 b) obtained the same inhibition for 6.8×10^{-7} M 2,4-D in solution culture (pH 6.8). Ready & Grant (1947) found seedlings of *Cucumis sativus* to be especially sensitive to 2,4-D; the growth of the primary roots was inhibited to 50 % by a 2.1×10^{-7} M solution of 2,4-D (unbuffered solution, filter paper method). For *Avena sativa*-seedlings grown on filter paper moistened with pure solutions of growth substances Lane (1936) found 1.7×10^{-6} M IAA necessary for 50 % inhibition of the root growth, while Bonner & Koepfli (1939) report 3×10^{-7} M for IAA and 7×10^{-6} for NAA. Using *Zea Mays* Swanson (1946) found the corresponding value for 2,4-D to be 2.4×10^{-6} M.

The sensitivity of the root growth inhibition test is strongly dependent upon the pH of the medium. For wheat roots in solution culture Marmer (1937) found that 6.8×10^{-8} M IAA gave 50 % growth inhibition at pH 4.6, while the same degree of inhibition was obtained with 1.0×10^{-4} M at pH 7.5. For *Avena sativa* Bonner & Koepfli (1939) report the corresponding values to be 3×10^{-7} M IAA at pH 4 and 1×10^{-5} M at pH 7. Working with *Lepidium* roots in 0.9×10^{-6} M 2,4-D Audus (1949 b) found a considerable decrease in the growth inhibition from pH 5 to 8.

As this pH-effect is presumably connected with a more rapid absorption of the undissociated molecules than of the ions, the dissociation constants of the different growth substances must be considered in comparing their effects at a certain pH-value. For IAA the pK-value is 4.75 (Bonner 1938), and the percentage of undissociated molecules at pH 5.9 consequently 6.6; for 2,4-D the corresponding values are 3.28 (Audus 1949 b) and 0.24 %. The higher activity of IAA than of 2,4-D in the root growth inhibition test may thus well be related to its lower dissociation degree and higher rate of penetration into the roots. For NAA no pK-value is available, but it seems probable that it should lie fairly close to that of IAA or of phenylacetic acid (4.3), and then the order of the three curves (NAA, 2,4-D, IAA) in figs. 1 and 2 can not be wholly explained on this basis. The slightly higher activity of the methyl ester of 2,4-D than of the acid (fig. 2) may also be related

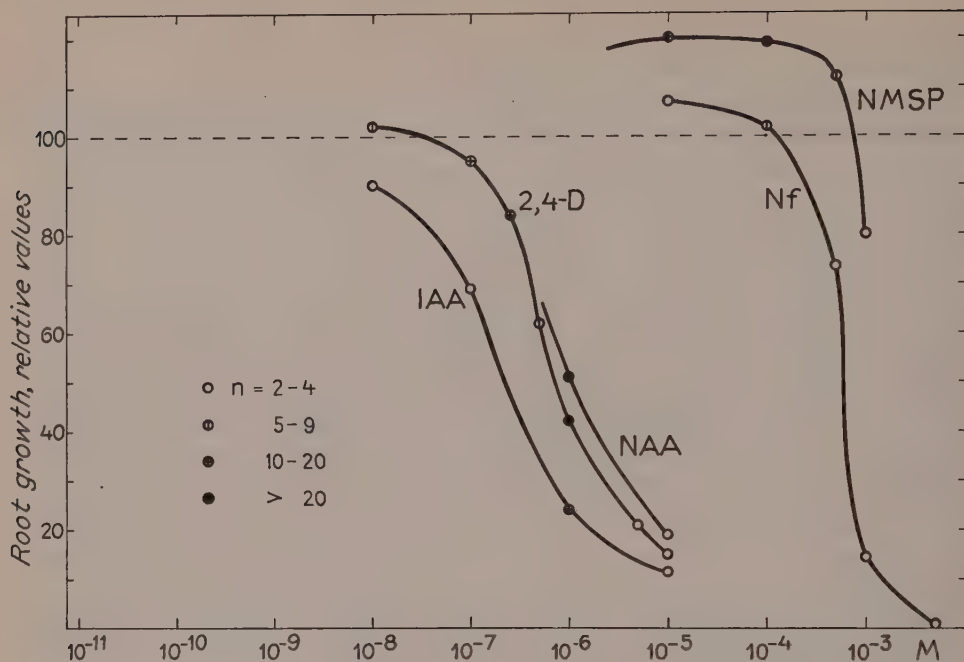


Fig. 1. Graphs showing the effects of different auxins and auxin antagonists in the *F*-test. Root growth in per cent of control plotted against a logarithmic concentration scale (M =mole per litre). n is the number of dishes, each with 20 seedlings, represented by the points.

to a more rapid absorption (cp. Stenlid 1949 p. 365) which is perhaps partly balanced by a lower activity of the ester within the root.

The results on the interaction of NAA and 1-naphthol (Nf) are presented in tables 2 and 3. In order to give an idea of the consistency of the effects obtained the average growth values for each dish with 20 seedlings or each tube with 14 seedlings are first shown. From the average values of each experimental series the relative growth values have then been computed by bringing the growth of the control seedlings to 100. To show the effect of Nf in the presence of NAA more clearly another series of relative values, starting from the pure NAA-solution, has been given in brackets. Both in the *F*-tests and in the *S*-tests a Nf concentration which alone causes only slight effects or a slight inhibition, will, in the presence of NAA, give consistent positive effects on the root growth. In the solutions containing Nf the roots assumed a violet tinge, mainly localized to the root hair zone.

The same type of results were obtained with NMSP and NAA (tables 4 and 5). Alone, the NMSP concentrations used gave a slight stimulation. In

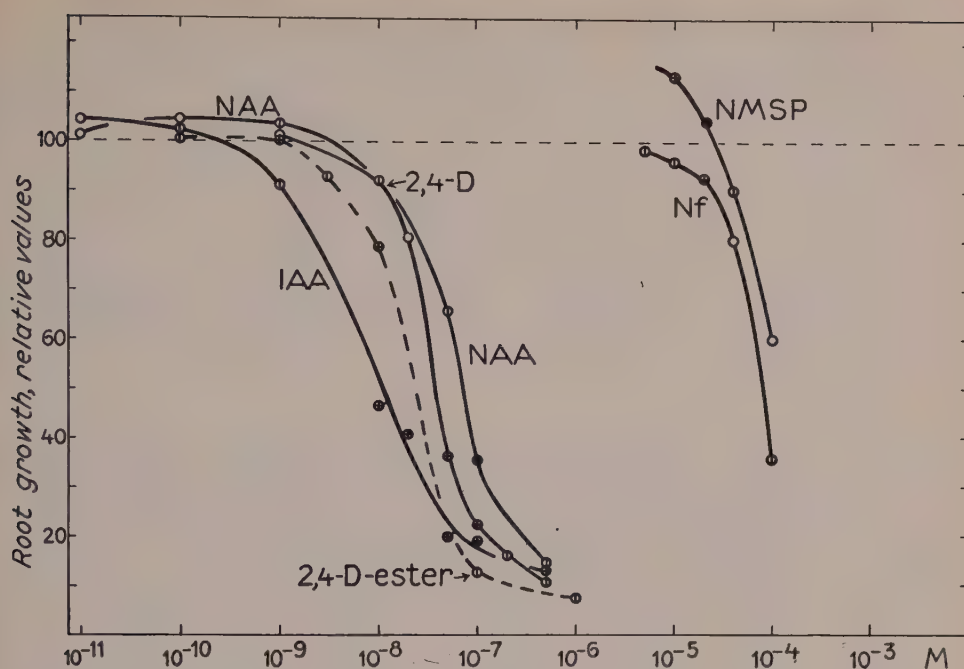


Fig. 2. Results of the S-tests, presented as in fig 1. Number of tubes, each with 14 seedlings, indicated as in fig. 1. The value for 10^{-8} M NAA is 92 % ($n=7$), i.e. identical with that for 2,4-D.

the presence of an inhibiting amount of NAA, however, the restoration of the root growth was very strong, the growth in the mixtures being almost as rapid and sometimes equal to that of the control roots. At a more strongly inhibiting concentration of NAA (table 6) the restoring effect of NMSP is lower. As, on the other hand, the growth in the mixtures will never exceed that of the controls to a very high degree, there will certainly be a medium NAA concentration giving an optimal growth restoration on addition of

Table 1. Molar concentrations needed for 50 per cent inhibition of the growth of *Linum* roots, pH=5.9.

Substance	F-test	S-test	F/S
NAA	10.6×10^{-7}	6.3×10^{-8}	16.8
2,4-D	7.1×10^{-7}	4.0×10^{-8}	17.8
2,4-D, methyl ester ...	—	2.3×10^{-8}	—
IAA	2.5×10^{-7}	1.1×10^{-8}	22.7
Nf	5.6×10^{-4}	8.5×10^{-5}	6.6

Table 2. *F-tests with NAA and 1-naphtol (Nf)*. Each of the absolute values represents the average growth of 20 roots. The exponential numbers denote the molar concentrations.

	Exp.	Control	10^{-4} Nf	10^{-6} NAA	10^{-6} NAA + 10^{-4} Nf
Growth, mm/19 h	1	{15.1	13.9	7.5	9.3
		{15.8	14.7	8.2	9.9
	2	{15.1	15.4	8.0	9.0
		{15.6	15.6	8.3	9.3
	3	{15.0	16.1	9.0	12.7
		{15.2	16.9	9.1	12.9
Growth, relative values	1	100	92	51 (100)	62 (122)
	2	100	101	53 (100)	60 (112)
	3	100	109	60 (100)	85 (141)

Table 3. *S-tests with NAA and 1-naphtol (Nf)*. Each of the absolute values represents the average growth of 14 roots.

	Exp.	Control	2×10^{-5} Nf	10^{-7} NAA	10^{-7} NAA + 2×10^{-5} Nf
Growth, mm/17 h	1	{26.3	24.2	8.0	9.8
		{27.1	24.4	8.1	10.1
		{27.5	24.8	8.3	10.2
	2	{25.8	23.0	7.9	10.3
		{25.9	24.1	8.5	10.8
		{27.2	25.2	9.2	10.8
Growth, rela- tive values	1	100	91	30 (100)	37 (123)
	2	100	92	32 (100)	40 (125)

NMSP. A comparison of tables 2 and 4 or of tables 3 and 5 will show that NMSP is much more effective than Nf in this respect.

As will be apparent from the results with 2,4-D and NMSP reported in tables 7—9, the type of interaction just described is not restricted to substances with the same nucleus. Quite contrary to such an expectation the restoring effect of NMSP seems to be greater for 2,4-D inhibited roots than for NAA inhibited ones. An exact comparison must be postponed, however, until data for further concentrations of these inhibitors have been obtained. From tables 8 and 9 it will be seen that the degree of 2,4-D-inhibition giving optimal growth restoration on addition of 2×10^{-5} M NMSP is perhaps above 80 %.

For roots inhibited by IAA the effect of NMSP (tables 10 and 11) is quite conspicuous though weaker than for those inhibited by NAA or 2,4-D. This difference is possibly significant as the comparatively slight growth restoration is apparent for strongly inhibited (table 10) as well as for moderately inhibited roots (table 11).

Table 4. *F-tests with NAA and NMSP*. Values presented as in table 2.

	Exp.	Control	10^{-4} NMSP	10^{-6} NAA	10^{-6} NAA + 10^{-4} NMSP
Growth, mm/19 h	1	{14.5	16.5	6.6	14.7
		{15.2	17.1	7.6	15.3
	2	{17.3	18.6	7.8	14.7
		{17.3	19.2	7.8	16.7
	3	{16.3	18.7	7.1	16.3
		{17.0	19.4	7.7	16.9
Growth, relative values	1	100	113	48 (100)	101 (211)
	2	100	109	45 (100)	91 (201)
	3	100	114	44 (100)	100 (224)

Table 5. *S-tests with NAA and NMSP*. Values presented as in table 3.

	Exp.	Control	NMSP		10^{-7} NAA + NMSP		
			10^{-5}	2×10^{-5}	0	10^{-5}	2×10^{-5}
Growth, mm/17 h	1 ¹	{23.2	28.7	27.8	6.9	20.3	20.1
		{25.7	28.8	28.4	9.6	21.0	21.8
	2	{23.7	24.2	—	8.1	16.4	—
		{23.8	27.0	—	8.4	19.5	—
	3	28.4	{ —	30.2	8.8	19.9	25.1
			{ —	30.6	11.8	22.3	
	4	{23.1	—	23.4	8.6	—	18.4
		{23.2	—	24.3	8.8	—	18.4
		{24.0	—	24.5	9.0	—	19.2
		{24.0	—	24.6	9.1	—	19.6
Growth, relative values	1	100	118	115	34 (100)	85 (251)	86 (254)
	2	100	108	—	35 (100)	76 (217)	—
	3	100	—	107	36 (100)	74 (205)	88 (244)
	4	100	—	103	38 (100)	—	80 (213)

¹ In exp. 1 NMSP was also used in the concentration 4×10^{-5} . The growth values were 23.4 and 24.6 mm or 98 %. In combination with 10^{-7} NAA the corresponding values were 19.0 and 19.8 mm or 79 (235) %.

Table 6. *S-tests with NAA and NMSP*. Values presented as in table 3.

	Exp.	Control	10^{-5} NMSP	5×10^{-7} NAA	5×10^{-7} NAA + 10^{-5} NMSP
Growth, mm/17 h	1	{24.7	26.4	3.6	5.4
		{26.7	26.4	3.8	6.0
	2	{23.7	24.2	3.2	4.7
		{23.8	27.0	3.4	5.1
Growth, re- lative values	1	100	103	14 (100)	22 (154)
	2	100	108	14 (100)	21 (149)

Table 7. *F-tests with 2,4-D and NMSP. Values presented as in table 2.*

	Exp.	Control	10^{-4} NMSP	10^{-6} 2,4-D	10^{-6} 2,4-D + 10^{-4} NMSP
Growth, mm/19 h	1	{16.6	18.3	4.6	14.1
		{16.6	18.4	5.4	16.1
	2	{14.1	19.1	5.1	16.9
		{15.3	19.9	6.1	17.4
	3	{16.0	19.0	6.5	16.2
		{16.5	19.2	6.8	16.4
Growth, relative values	1	100	111	30 (100)	91 (302)
	2	100	133	38 (100)	117 (306)
	3	100	117	41 (100)	100 (245)

Table 8. *S-tests with 2,4-D and NMSP. Values presented as in table 3.*

	Exp.	Control	NMSP		10^{-7} 2,4-D + NMSP		
			2×10^{-5}	4×10^{-5}	0	2×10^{-5}	4×10^{-5}
Growth, mm/17 h	1	{28.2	23.5	22.1	6.7	22.1	22.2
		{28.5	27.3	24.3	6.8	24.2	22.6
	2	{22.3	21.5	19.8	4.8	18.6	17.7
		{22.5	21.1	21.1	4.9	18.6	18.1
	3	{27.5	28.7	—	5.7	24.2	—
		{29.2	28.9	—	5.8	24.6	—
Growth, relative values	1	100	90	82	24 (100)	82 (343)	79 (332)
	2	100	96	91	22 (100)	83 (383)	80 (369)
	3	100	100	—	20 (100)	85 (412)	—

Table 9. *S-tests with 2,4-D and NMSP. Values presented as in table 3.*

	Exp.	Control	2×10^{-5} NMSP	5×10^{-8} 2,4-D	5×10^{-8} 2,4-D + 2×10^{-5} NMSP
Growth, mm/17 h	1	{22.4	20.1	6.8	20.8
		{23.7	20.2	7.1	21.1
		{22.6	22.6	7.2	20.0
	2	{24.0	23.0	7.4	20.7
		{24.6	23.4	8.4	21.6
Growth, rela- tive values	1	100	87	30 (100)	91 (301)
	2	100	97	32 (100)	88 (270)

In order to facilitate a survey of the results obtained with NMSP in combination with different growth substances the average growth values of tables 4—11 have been compiled in table 12.

For the sake of comparison it was thought appropriate to test a substance known as an auxin synergist also. For this purpose 2,3,5-triodobenzoic acid (TIBA) was used, a substance which was first announced as an auxin anta-

Table 10. *F*-tests with IAA and NMSP. Values presented as in table 2.

	Exp.	Control	NMSP		10 ⁻⁶ IAA + NMSP		
			10 ⁻⁴	5 × 10 ⁻⁴	0	10 ⁻⁴	5 × 10 ⁻⁴
Growth, mm/19 h	1	{ 16.9	18.6	—	3.2	7.6	—
		{ 17.8	18.6	—	3.9	7.8	—
	2	{ 14.8	16.9	—	3.2	4.7	—
		{ 16.2	17.3	—	3.5	4.9	—
	3	{ 12.7	15.7	14.5	3.2	4.2	5.7
		{ 13.3	16.8	15.6	3.6	4.6	6.2
Growth, relative values	1	100	107	—	20 (100)	44 (217)	—
	2	100	110	—	22 (100)	31 (143)	—
	3	100	125	116	26 (100)	34 (129)	46 (175)

Table 11. *S*-tests with IAA and NMSP. Values presented as in table 3.

	Exp.	Control	2 × 10 ⁻⁵ NMSP	2 × 10 ⁻⁸ IAA	2 × 10 ⁻⁸ IAA + 2 × 10 ⁻⁶ NMSP
Growth, mm/17 h	1	{ 24.6	25.0	7.8	16.5
		{ 26.6	25.5	9.3	16.7
	2	{ 25.2	25.2	10.2	14.9
		{ 25.4	26.3	10.4	16.4
	3	{ 26.8	26.0	11.1	17.7
		{ 14.2	23.2	6.9	11.5
Growth, relative values	1	100	99	33 (100)	65 (193)
	2	100	100	41 (100)	63 (154)
	3	100	141	49 (100)	78 (160)

Table 12. *The interaction of NMSP with various growth substances. Summary of fig. 1—2 and tables 4—11. Relative growth values (control=100). The values in brackets represent the growth in presence of NMSP, calculated as per cent of the growth value in buffer solution with growth substance only. A=»activity coefficients» of the different growth substances in the presence of 2 × 10⁻⁵ M NMSP, calculated as described on p. 458.*

		No growth subst. added	2,4-D		NAA		IAA
			10 ⁻⁶ M		10 ⁻⁶ M		10 ⁻⁶ M
<i>F</i> -tests	No NMSP	100	36		46		23
	10 ⁻⁴ M NMSP ...	119	103 (284)		97 (212)		36 (163)
<i>S</i> -tests			10 ⁻⁷	5 × 10 ⁻⁸	5 × 10 ⁻⁷	10 ⁻⁷	2 × 10 ⁻⁸
	No NMSP	100	22	31	14	36	41
	2 × 10 ⁻⁵ M NMSP	104	83 (379)	90 (286)	22 ¹ (152)	85 ² (237)	69 (169)
	A		0.18	0.26	0.40 ¹	0.20	0.22

¹ 10⁻⁵ M NMSP.² Corresponding values for 10⁻⁵ M NMSP 78 (224), A=0.28.

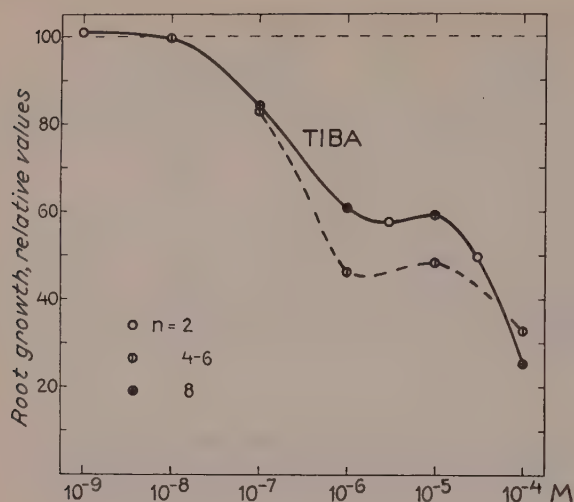


Fig. 3. Effects of TIBA upon root growth (S-test). TIBA alone or in the presence of 3×10^{-9} M IAA (broken line). n is the number of tubes, each with 14 seedlings, represented by the points.

gonist and a florigenic substance, but has since been shown to enhance the effect of IAA in split pea internodes and in *Avena* coleoptiles under other conditions (Thimann & Bonner 1948). The action curve of TIBA obtained in the S-test (fig. 3) is of a very peculiar type with almost identical degree of root inhibition for 10^{-6} and 10^{-5} M, a phenomenon which reappeared in four different series of experiments. If we accept the hypothesis of a supra-optimal auxin concentration in the roots, the first part of the curve (10^{-8} — 10^{-6} M) could possibly be explained as a synergistic effect upon the native auxin, while the second decline (10^{-5} — 10^{-4} M) could be related to a less specific toxic effect upon the plasm, or perhaps to an antagonistic effect upon the auxin. In the presence of a slightly inhibiting concentration of IAA (3×10^{-9} M, root growth 70 %) a curve of the same type is obtained (broken line in fig. 3, growth in IAA alone brought to 100), only the first slope to 10^{-6} M TIBA being steeper, a result which is compatible to the explanation tentatively suggested.

Discussion

Several substances have earlier been characterized as auxin antagonists. In 1939 Voss accumulated some data indicating the presence of a native auxin antagonist in the endosperm of corn seedlings which normally depresses the growth of the mesocotyl and stimulates the root growth. Skoog, Schneider & Malan (1942) suggested that γ -phenylbutyric acid (one of Went's hemiauxins) acts as a competitive auxin antagonist in the standard *Avena* test and in the straight growth of the coleoptiles. The observed effects cannot

be explained without additional hypotheses, however, and further experiments are needed for the elucidation of the action of this substance (cp. Veldstra & Booij 1949 p. 300). The hypothesis that 2,4-dichlorophenoxyacetic acid (2,4-D) and related substances act by releasing native auxin from inactive protein complexes (Skoog 1947 p. 545, 557) should also be mentioned. Bonner (1949 p. 329) found that 2,4-dichloranisole ($C_6H_3Cl_2 \cdot O \cdot CH_3$) depresses the auxin-induced growth of *Avena* sections, but does not act as an ideal antagonist. Antagonistic effects of 2,4-dichlorophenoxyethylamine ($C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot CH_2 \cdot NH_2$) on the action of 2,4-D (Jones, Metcalfe & Sexton 1949, rape roots) or of *naphthalenemethanesulfonic acid* on the action of NAA (Veldstra & Havinga 1943 p. 846, pea test) have been sought for, but with negative results.

An interesting group of growth inhibitors which have sometimes been called auxin antagonists (e.g. Moewus 1949 a), is the unsaturated lactones of the *coumarin* type. As Larsen (1947 p. 354) has pointed out there are, however, no indications that these substances are true auxin antagonists, but rather that they control another step of the growth process. Thus, they retard root growth in all concentrations, and the effects of for example coumarin and IAA upon root growth are purely additive (Moewus 1949 a p. 62).

Triiodobenzoic acid (TIBA) has been found to antagonize the effects of IAA in *Avena* coleoptiles when present in high mol-ratios (Galston 1947, Waard & Florschütz 1948), but to enhance its effects in lower mol-ratios (Thimann & Bonner 1948). If the interpretation of the experiments here reported (p. 456) is correct, the synergistic effect should be prevailing to much higher mol-ratios in the roots than in shoots.

Quite recently Burström (1950) has found α -(*p*-chlorophenoxy)-isobutyric acid [$C_6H_4Cl \cdot O \cdot C(CH_3)_2 \cdot COOH$] (PCIB) to act as an anti-auxin in wheat roots. He assumes two independent, locally separated auxin actions in the elongation of the root cells, and finds an anti-auxin action of PCIB in both phases. In the present connection we need only discuss the overall process of longitudinal growth of roots which is naturally the complex outcome of cell divisions, cell elongation rates and final cell lengths. As to the root elongation Burström reports an initial increase by 60 % and more in the presence of 10^{-6} M PCIB, an effect which is considerably stronger than that of NMSP upon *Linum* roots. Restricting ourselves to the final outcome of the root growth processes the results with PCIB as well as those with NMSP (figs. 1 and 2) can thus be said to support the hypothesis of a supraoptimal auxin concentration in normal roots.

Positive effects of auxin on root elongation naturally represent a strong indication against such a hypothesis. Such effects are, however, mainly

reported for isolated or decapitated roots, where the concentration of native auxin could be expected to be lower than normal. In other cases it seems to be the question of adaptation phenomena which can perhaps be at work also in causing the slight positive effects shown in fig. 2 as well as in the experiments of Moewus (1949 a, b) who reports about 12 % stimulation for 10^{-10} to 10^{-11} M IAA (Lepidium seedlings on filter paper).

The effects of NMSP when applied together with different growth substances are summarized in table 12. As already pointed out the restorative effect of NMSP upon root growth varies with the degree of inhibition caused by the growth substance alone, and probably passes a maximum value at medium inhibition. A possible way for the quantitative comparison of the antagonistic effects obtained is perhaps to evaluate the »activity» of a growth substance when present together with the antagonist from the actual growth rate and figs. 1 and 2. If, for example, the relative growth rate in 10^{-7} M 2,4-D + 2×10^{-5} M NMSP is 83 % (table 12), it follows from fig. 2 that the »activity» of 2,4-D in this mixture is 1.8×10^{-8} and the »activity coefficient» consequently 0.18. The coefficients thus obtained for the S-tests are presented in table 12, but further experiments are needed before their quantitative significance can be judged with certainty. For 10^{-7} M NAA we find an »activity coefficient» of 0.9 in the presence of 2×10^{-5} M Nf (table 3). The growth values for pure NAA in these experiments are somewhat lower than the average value of fig. 2 (35.5 %), and a correction for this deviation changes the coefficient to about 0.7. The difference between NMSP and Nf is thus quite obvious. A comparison with Burströms (1950, table 2) data on the effect of PCIB on the final cell length in wheat roots (proportional to the elongation of the roots) is not possible because of differences in methods and material.

As to the possible mechanisms of auxin antagonism and synergism not much can be said with certainty. An impressive scheme, based upon an enzyme theory of the auxin action, was first proposed by Skoog, Schneider & Malan (1942). Veldstra, on the other side, thinks a co-enzyme action in the chemical sense improbable and lays more stress upon a possible physico-chemical type of action in plasmatic membranes or within the protoplasm itself, resulting in a regulating action upon enzyme systems (Veldstra 1944, Veldstra & Booij 1949). Also the possibility of a more general action upon protoplasmic structure has to be taken into consideration (Northen 1942, Jones 1946, Audus 1949 a p. 78). It is conceivable that actions upon specific parts of the protoplasmic structure should lead to highly specific results, among other things by influencing the biochemical processes which are governed by the genes.

In respect to the attachment of the auxin molecules or ions to the specific

positions necessary for their action special stress has been laid on the basal ring system (Veldstra 1944). The experiments with 1-naphthol here reported give a certain support for this hypothesis, but the much more pronounced competitive action of NMSP suggests that the type of the side chain is perhaps also of importance in determining the affinity of the molecules for these positions. Which properties of the side chain are responsible for the fact that NMSP antagonizes different growth substances, and is not an auxin in itself or simply inactive, can not yet be settled. The sulfur bridge is perhaps of importance (cp. the oxygen bridge in PCIB) though not sufficient in itself, and the short side-branch ($-\text{CH}_3$) is not fundamental as recent tests with 1-naphthylmethylsulfideacetic acid have shown this substance to have the same type of antagonistic activity.

The problem is further complicated by the existence of auxin synergists, perhaps of different types. Went (1939) found some substances (γ -phenylbutyric acid, cyclohexane-acetic acid, etc.) which, though in themselves without auxin activity, were able to increase the sensitivity of f.ex. the pea test for auxin, and assumed that they were active in a »preparatory reaction» which is followed by the growth reaction proper. Veldstra & Booij (1949) added further substances (naphthalenebutyric acid, decahydro-naphthalene-acetic acid, di-n-amylacetic acid, etc.) with strong synergistic effects in the pea test, and suggest that these substances, having an increased lipophilic character, are absorbed preferably to the plasmic membranes, thus preventing the absorption of the auxin molecules in inactive positions and enabling them to reach the proper places in the plasm more efficiently. The experiments on the effects of TIBA upon root growth give no new material for the explanation of the mechanism of the synergistic action. Further studies on the effects of »hemiauxines» now under way seem to show that there are synergists of different types. Thus, γ -phenylbutyric acid shows an activity curve in the S-test which is of the normal sigmoidal form, but occupies a position intermediate between NMSP and NAA.

Investigations on the effects of NMSP in shoots are also under way. In the leaf repression test, made as described by Brown & Weintraub (1950), the effects of 2,4-D can be eliminated by the use of NMSP in a high molar ratio. The effects of NMSP in the Avena cylinder test and in the pea test are studied by Mr. E. Hesselman. The cylinder test has given results of the type expected, while it seems more difficult to obtain consistent results with the pea test.

Summary

Young flax seedlings have been grown on moist filter paper (*F*-tests) or in solution culture (*S*-tests). The effects upon root elongation of various

concentrations of indole-3-acetic acid (IAA), naphthalene-acetic acid (NAA), and of 2,4-dichlorophenoxyacetic acid (2,4-D) are shown in figs. 1 and 2. The *S*-test is about 20 times more sensitive than the *F*-test.

When an amount of 1-naphthol (Nf) which is by itself slightly inhibiting or without consistent effects on the root growth, is added to an inhibiting concentration of NAA, a slight but consistent restoration of the growth is apparent. Much stronger effects of the same type can be obtained by the use of α -(1-naphthylmethylsulfide)-propionic acid (NMSP) which substance is effective in restoring the growth of roots inhibited by NAA as well as by IAA or 2,4-D. In lower concentrations of NMSP alone the root growth is increased by 15–20 % over that of the control cultures.

It is suggested that Nf and NMSP act as competitive auxin antagonists. The stimulation of the roots growing without external auxin application must then be due to lowered activity of the native auxin which is normally present in a supra-optimum concentration. Experiments with 2,3,5-triiodobenzoic acid (TIBA), known to act as an auxin synergist under some conditions, gave results (fig. 3) which seem compatible to the explanation just suggested.

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References

- Audus, L. J.: The mechanism of auxin action. — *Biol. Reviews* 24: 51. 1949 (a).
 — Studies on the pH-relationship of root growth and its inhibition by 2:4-dichlorophenoxyacetic acid and coumarin. — *New Phytol.* 48: 97. 1949 (b).
 Bonner, D. M.: Relation of environment and of the physical properties of synthetic growth substances to the growth reaction. — *Bot. Gaz.* 100: 200. 1938.
 Bonner, J.: Limiting factors and growth inhibitors in the growth of the *Avena* coleoptile. — *Amer. Jour. Bot.* 36: 323. 1949.
 — & Koepfli, J. B.: The inhibition of root growth by auxins. — *Amer. Jour. Bot.* 26: 557. 1939.
 Brown, J. W. & Weintraub, R. L.: A leaf-repression method for evaluation of formative activity of plant growth-regulating chemicals. — *Bot. Gaz.* 111: 448. 1950.
 Burström, H.: Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
 Galston, A. W.: The effect of 2, 3, 5-triiodobenzoic acid on the growth and flowering of soybeans. — *Amer. Jour. Bot.* 34: 356. 1947.
 Jones, R. F.: Factors involved in the reaction between plant cells and substances affecting growth. — *New Phytol.* 45: 169. 1946.
 Jones, R. L., Metcalfe, T. P. & Sexton, W. A.: The relationship between the constitution and the effect of chemical compounds on plant growth. 1. 2-phenoxyethylamine derivatives. — *Biochem. Jour.* 45: 143. 1949.

- Lane, R. H.: The inhibition of roots by growth hormone. — *Amer. Jour. Bot.* 23: 532. 1936.
- Larsen, P.: Avena curvatures produced by mixtures of growth promoting and growth inhibiting substances. — *Amer. Jour. Bot.* 34: 349. 1947.
- Marmer, Dina R.: Growth of wheat seedlings in solutions containing chemical growth substances. — *Amer. Jour. Bot.* 24: 139. 1937.
- Moewus, F.: Die Wirkung von Wuchs- und Hemmstoffen auf die Kressewurzel. — *Biol. Zentralbl.* 68: 58. 1949 (a).
- Der Kressewurzeltest, ein neuer quantitativer Wuchsstofftest. — *Biol. Zentralbl.* 68: 118. 1949 (b).
- Northern, H. T.: Relationship of dissociation of cellular proteins by auxins to growth. — *Bot. Gaz.* 103: 668. 1942.
- Ready, D. & Grant, V. Q.: A rapid sensitive method for determination of low concentrations of 2,4-dichlorophenoxyacetic acid in aqueous solution. — *Bot. Gaz.* 109: 39. 1947.
- Skoog, F.: Growth substances in higher plants. — *Ann. Rev. Biochem.* 16: 529. 1947.
- , Schneider, C. L. & Malan, P.: Interactions of auxins in growth and inhibition. — *Amer. Jour. Bot.* 29: 568. 1942.
- Stenlid, G.: The effect of 2,4-dinitrophenol upon oxygen consumption and glucose uptake in young wheat roots. — *Physiol. Plant.* 2: 350. 1949.
- Swanson, C. P.: A simple bio-assay method for the determination of low concentrations of 2,4-dichlorophenoxyacetic acid in aqueous solutions. — *Bot. Gaz.* 107: 507. 1946.
- Thimann, K. V.: Plant growth hormones. — Pincus & Thimann, *The hormones* 1: 5. New York 1948.
- & Bonner, W. D.: The action of tri-iodobenzoic acid on growth. — *Plant Physiol.* 23: 158. 1948.
- Veldstra, H.: Researches on plant growth substances. IV—V. Relation between chemical structure and physiological activity. I—II. — *Enzymologia* 11: 97, 137. 1944.
- & Booij, H. L.: Researches on plant growth regulators. XVII. Structure and activity. On the mechanism of the action. III. — *Biochim. et Biophys. Acta* 3: 278. 1949.
- & Havinga, E.: Untersuchungen über pflanzliche Wuchsstoffe. VII. Über Struktur und Wirkungsmechanismus der pflanzlichen Wuchs- und Hemmstoffe. — *Rec. Trav. Chim. Pays-Bas* 62: 841. 1943.
- Voss, H.: Nachweis des inaktiven Wuchsstoffes, eines Wuchsstoffantagonisten und deren wachstumsregulatorische Bedeutung. — *Planta* 30: 252. 1939.
- Waard, Jeanne de & Florschütz, P. A.: On the interaction of 2,3,5-triiodobenzoic acid and indole-3-acetic acid in growth processes. — *Kon. Nederl. Akad. Wet., Proc.* 51: 1317. 1948.
- Went, F. W.: Further analysis of the pea test for auxin. — *Bul. Torrey Bot. Club* 66: 391. 1939.

Zur Erklärung der Ultrafilterwirkung der Plasmahaut

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Einleitung

Die ausgesprochene Durchlässigkeit des Protoplasmas für kleinmolekulare Verbindungen verbleibt immer noch die Achillesferse der als fast unversehrter Sieger durchgekommenen Overtonschen Lipoidhypothese. Danielli (Davson und Danielli 1943) hat es freilich versucht, mit einem entschlossenen Schnitt die ganze kranke Stelle zu entfernen, indem er erklärt, dass die Molekülgrösse überhaupt nicht zu den die Permeationsgeschwindigkeit beeinflussen den Faktoren gehört. Tatsache ist jedoch, dass es manchmal kaum zu vermeiden ist, sich der »Ultrafilterwirkung« doch wenigstens als eine Art Hilfsbegriff zu bedienen, dessen physikalische Erklärung man klugerweise übergehen kann. So z.B. kamen Collander und Wikström (1949) zu dem Ergebnis, dass »irgendeine Art von einer Molekülsiebwirkung mit im Spiele ist«, fügen jedoch vorsichtigerweise hinzu, dass »damit aber durchaus nicht etwa behauptet werden soll, dass es sich um permanente, wassergefüllte Poren in der Plasmahaut handeln muss«. (Vgl. auch Collander 1949.)

Beim Erwägen der Resultate meiner mit kleinmolekularen Stoffen ausgeführten Versuche (Wartiovaara 1944, 1949), ist in mir allmählich der Verdacht aufgekommen, dass die Molekülgrösse bei der Suche nach den Ursachen der Ultrafilterwirkung der Plasmahaut tatsächlich eine etwas einseitige Berücksichtigung gefunden hat. Demgegenüber ist dem *Bau* der permeierenden Moleküle merkwürdig wenig Beachtung geschenkt worden. Doch ist man über die Eigenschaften der Moleküle als dreidimensionale Körper ziemlich eingehend unterrichtet, insoweit es sich um die zur Standardausrüstung des Permeabilitätsforschers gehörenden einfachen Stoffe han-

delt. In der vorliegenden Untersuchung werden die von diesem bis jetzt noch fast unausgenutzten Ausgangspunkt dargebotenen Möglichkeiten zum Verständnis der Ultrafilterwirkung der Plasmahaut, zur Hauptsache auf Grund von Permeationsversuchen mit pflanzlichen Zellen, einer Betrachtung unterzogen.

Die zwei Hauptformen der "Ultrafilterwirkung"

Ist das Wesen eines materiellen oder imaginären Ultrafilters nicht sicher bekannt, wie es sich im Falle der Plasmahaut verhält, so mag man ebenso gut von einer herabgesetzten Permeabilität für grossmolekulare Stoffe reden, wie von einer erhöhten Permeabilität für kleinmolekulare Stoffe. Es ist ja keineswegs ohne weiteres klar, in welcher Richtung sich die mittlere Permeabilität verschieben würde, wenn es möglich wäre, die Ultrafilterwirkung auszuschalten. Da aber die Auffassung tief eingebürgert zu sein scheint, dass die Permeabilität der Plasmahaut zumal für kleinere Moleküle bei Abwesenheit der Ultrafilterwirkung wohl immer niedriger wäre, so werde ich die Permeation der kleinsten Moleküle üblicherweise als beschleunigt bezeichnen und den Ausdruck Ultrafilterwirkung für die Ursachen einer beschleunigten Permeation überhaupt gebrauchen.

Die Ultrafilterwirkung in jenem Sinne ist keine einheitliche Erscheinung, sondern lässt sich in zwei deutlich verschiedene Formen aufteilen. In der Tat dürften die beiden Formen unter sich sehr wenig Gemeinsames besitzen und verdienen daher provisorische Sondernamen.

Mit dem Wort *Siebwirkung* wird im nachstehenden die Erscheinung gemeint, dass kleinmolekulare Stoffe ein grösseres Permeationsvermögen aufweisen, als auf Grund ihrer Lipoidlöslichkeit zu erwarten wäre. Des weiteren wird vorausgesetzt, dass es sich um eine Permeation von rein diffusionsartigem Charakter und nicht um eine mit dem aktiven Energiehaushalt der Zelle verknüpfte adenoide Tätigkeit handelt. Diese Ultrafilterwirkung in bezug auf kleinmolekulare Stoffe äussert sich gleichartig bei den verschiedensten Zelltypen, abgesehen von solchen wie *Beggiatoa* und *Oscillatoria*, denen eine eigentliche Plasmahaut anscheinend fehlt (vgl. Drawert 1949). Nach diesem ihrem generellen Charakter zu schliessen, hat man es mit einer prinzipiell einfachen Erscheinung zu tun, die sich eng an das vermutlich für alle Zellen gemeinsame Grundschemata anschliesst, auf welchem der ausserordentlich grosse diffusionshemmende Effekt der Plasmahaut beruht.

Die andere Form der Ultrafilterwirkung, die sich in einer bedeutend hohen Zuckerpermeabilität zumal bei den Diatomeen äussert (Höfler 1940), dürfte im Gegensatz zu der Siebwirkung mit der physiologischen Spezialisierung der Zelle und den dieser entsprechenden mehr oder minder komplizierten

Strukturzügen der Plasmahaut zusammenhängen. Sie mag in weniger deutlicher Ausbildung, der Siebwirkung aufgelagert, ganz allgemein vorkommen. Ist es doch wahrscheinlich, dass in der Plasmahaut aller Zellen gewisse Stellen vorhanden sind, die speziell dem aktiven Stoffaustausch dienen und deren isolierende Eigenschaften schwach ausgeprägt sein können.

Ein Grund dafür, dass die Frage von der »Ultrafilterwirkung« so verworren erscheint, dürfte darin zu erblicken sein, dass die beiden Formen dieser Wirkung nicht streng genug auseinandergehalten worden sind. Ich möchte zur Unterscheidung von der Siebwirkung die Bezeichnung *Porenpermeabilität* für die letzterwähnte Form der Ultrafilterwirkung gebrauchen, und zwar in rein deskriptivem Sinne: als ob die Permeation zum Teil durch Poren hindurch stattfindet.

Weder die Siebwirkung noch die Porenpermeabilität für grossmolekulare, sehr hydrophile Stoffe setzen die Existenz eines festen Porengerüsts voraus, sondern eher einen mit dem einer Flüssigkeit vergleichbaren Zustand der Plasmahaut. Wäre es doch anderenfalls schwer zu verstehen, dass sie sich als positive Abweichungen von der Proportionalität zur Lipoidlöslichkeit äussern, während klare negative Abweichungen, d.h. herabgesetzte Permeabilität für grossmolekulare lipoidlösliche Stoffe, anscheinend fehlen. Gegen die Flüssigkeitsnatur der Plasmahaut dürften denn auch keine ernstlichen Einwände bestehen. Die Plasmahaut kann im Prinzip recht wohl ähnlich beschaffen sein wie die von Fettsäuren u. dgl. m. auf einer Wasseroberfläche gebildeten »zweidimensionalen Flüssigkeiten«. Insoweit sind sich wohl die meisten Physiologen einig. Das Wesen der hypothetischen »Poren« gibt dagegen dauernd Anlass zu Meinungsverschiedenheiten. An eine wirklich porige Flüssigkeit zu denken, scheint ohne weiteres ausgeschlossen, doch keine einzige von den zur Umgehung dieser Schwierigkeit bisher vorgebrachten Hypothesen hat vorläufig allgemeine Anerkennung gefunden.

Die Siebwirkung in bezug auf eine homologe Reihe

Einer von den schwerwiegendsten Beweisen, auf die man sich zugunsten der realen Existenz einer Siebstruktur der Plasmahaut berufen kann, ist die Tatsache, dass in aliphatischen homologen Reihen das Permeationsvermögen des ersten Gliedes oft deutlich grösser als das des zweiten Gliedes ist, trotzdem die Stoffe der betreffenden Reihe sonst der üblichen Regel folgen, welcher gemäss das Permeationsvermögen mit der zunehmenden Zahl der Kohlenstoffatome wächst. Es scheint also dem Durchmesser von »Poren« irgendwelcher Art ein bestimmter kritischer Moleküldurchmesser zu entsprechen.

Tabelle 1. Das Permeationsvermögen der Alkohole und Amide in Beziehung zu ihrer Lipoidlöslichkeit. P=Permeationsvermögen, k=Verteilungskoeffizient. (Nach Collander und Bärlund 1933; Collander 1949; Wartiovaara 1949.)

	P cm/st	Olivenöl		Äthyläther	
		k	P/k	k	P/k
Nitella clavata, 0° C					
Methanol	0.306	0.0063	50	0.14	2.2
Ethanol	0.280	0.032	8.8	0.26	1.1
Propanol	0.444	0.12	3.7	1.9	0.23
Butanol	1.07	0.56	1.9	7.7	0.14
Chara ceratophylla, 20—25° C					
Formamid	0.077	0.00076	100	0.0014	55
Azetamid	0.053	0.00083	64	0.0025	21
Propionamid	0.13	0.0036	36	0.013	10
Butyramid	0.17	0.0095	18	0.058	2.9

Die Siebwirkung braucht sich aber nicht ausschliesslich auf diejenigen Fälle zu beschränken, in denen ein niederes Homolog *absolut rascher* als das folgende höhere permeiert. Sie kann sich auch in Form einer *relativ verminderten Differenz* zwischen den Permeationsgeschwindigkeiten äussern. Wir können uns als Mass der Siebwirkung des Verhältnisses P/k bedienen, welches die Grösse der Abweichung von der Proportionalität zwischen Lipoidlöslichkeit und Permeabilität zum Ausdruck bringt, wenn P die Permeationskonstante und k den Verteilungskoeffizienten bedeuten. Wäre die Siebwirkung lediglich auf das erste Glied der homologen Reihe beschränkt, so wäre das Verhältniss nur in bezug auf dieses Glied grösser, für die übrigen dagegen konstant. So verhält es sich indessen nicht, sondern *die Siebwirkung scheint eine fortlaufende Funktion der Anzahl der Kohlenstoffatome darzustellen* (Tab. 1). Ihr zahlenmässiger Wert sinkt sowohl in der Alkohol- als in der Amidreihe jedenfalls bis zum vierten Glied. Die auffallende Umkehrung der Ordnungsfolge der niedrigsten Homologe in der Permeabilitätsreihe kommt dadurch zustande, dass *die Siebwirkung im Anfang der homologen Reihe relativ steiler fällt, als die Lipoidlöslichkeit steigt*.

Neben der qualitativ gleichartigen, fortlaufenden Abnahme lassen die vier P/k-Reihen auch augenfällige Unterschiede erkennen. Vor allem ist es bedenklich, dass ihre Bestufung sich verschieden gestaltet, je nachdem, ob die Olivenöl- oder die Ätherlöslichkeit als Mass der Lipoidlöslichkeit eingesetzt wird, während die tatsächliche Siebwirkung des Protoplasmas eindeutig definierbar sein müsste. Können auf sie überhaupt andere Schlussfolgerungen begründet werden, als dass sich die Siebwirkung zumindest bis zum vierten Glied der Reihen erstreckt?

Die Permeabilität ist eigentlich nicht direkt abhängig von der Lipoidlös-

lichkeit. In Wirklichkeit hängen ja beide von der Umwandlung der freien Energie ab, die beim Übertritt des gelösten Stoffes aus dem Wasser in die Lipoide der Plasmahaut bzw. in ein lipoidartiges Lösungsmittel stattfindet (Davson und Danielli 1943; Davies 1950). Ist der Energieumsatz an der Grenzfläche der Plasmahautlipoide einerseits und der des betreffenden Lösungsmittels andererseits gleich gross, was sich in einer allgemeinen Proportionalität zwischen der Permeabilität und dem Verteilungskoeffizienten äussert, so mag der Verteilungskoeffizient k als Mass der »Lipoidpermeabilität« und P/k als Mass der Siebwirkung gelten. Dies ist aller Wahrscheinlichkeit nach der Fall mit den aus der Olivenöllöslichkeit berechneten P/k -Werten der Alkoholreihe. Erstens ist die Permeabilität der naheverwandten Chara im grossen und ganzen der Olivenöllöslichkeit proportional, wie auch die Plasmahautlipoide und das Olivenöl grossenteils Derivate derselben Fettsäuren sein dürften. Zweitens verändert sich der Verteilungskoeffizient ziemlich regelmässig exponential mit zunehmender Anzahl der Kohlenstoffatome, ein Beweis dafür, dass keine von der Eigenart des Lösungsmittels abhängigen Faktoren die Löslichkeit wesentlich beeinflussen. Die übrigen k -Reihen zeigen Anomalien, deren Ursache nicht sicher bekannt ist, es dürfte sich jedenfalls um Faktoren handeln, die wenig mit der Permeabilität zu tun haben. Man könnte z.B. bezüglich der Amide die in Olivenöl enthaltene Ölsäure als einen solchen Faktor vermuten (vgl. Collander und Bärland 1933).

Die Zerlegung des Diffusionswiderstandes der Plasmahaut in nur zwei Komponenten ist eine rein mathematische Prozedur. In Wirklichkeit dürfte man es hierbei mit einer ganzen Anzahl von verschiedenen Faktoren zu tun haben. Doch, handelt es sich um die Glieder ein und derselben homologen Reihe, zumal um ihrem Molekülbau nach so einfache und sowohl chemisch als physiologisch so indifferente Stoffe, wie es die niederen Alkohole sind, so können wir allenthalben mit ziemlich grosser Zuversicht für diese zusätzlichen Faktoren in den verschiedenen Fällen eine gleichlautende Wirkung annehmen. Sie dürften somit die relativen Zahlenwerte der Permeabilität kaum in nennenswerterem Masse beeinflussen.

Nehmen wir also an, dass das Permeationsvermögen der Glieder der Alkoholreihe neben unbekannten konstanten Faktoren von zwei realen Variablen bestimmt wird: der Lipoidlöslichkeit, die in erster Linie als ein Energiefaktor anzusprechen ist, und der Siebwirkung, deren Wesen es zu klären gilt. Die uns als Ausgangspunkt zur Verfügung stehenden Fakta sind die folgenden.

1. Bezeichnet man den der Lipoidlöslichkeit proportionalen hypothetischen Grundwert der Permeabilität mit L und die Siebwirkung mit s , so ist die Permeabilität der Zelle

$$P = s \cdot L \quad (1)$$

Dies folgt schon daraus, dass wir die Siebwirkung als eine Abweichung von der Proportionalität zwischen der Lipoidlöslichkeit und der Permeabilität definierten und das Vergleichslipoid so zu wählen versuchten, dass k und L direkt proportional sind, dass also

$$s = P/k = P/L \quad (2)$$

2. Die Siebwirkung kann sich auch fast quantitativ ähnlich in verschiedenen homologen Reihen (Alkohole, Amide) äussern, trotz eines bedeutenden Unterschiedes in der Lipoidlöslichkeit und dem Permeationsvermögen der analogen Glieder der betreffenden Reihen.

3. Die Siebwirkung ist in der Alkoholreihe annähernd dem Ausdruck n^2 umgekehrt proportional, wenn die Zahl der Kohlenstoffatome mit n bezeichnet wird.

Auf Grund der Momente 1 und 2 kann der Schluss gezogen werden, dass nicht nur die höheren, sondern *auch die ersten Glieder jener homologen Reihen wesentlich dem Löslichkeitsprinzip gemäss permeieren*, während der Faktor s die Permeation derart beeinflusst, wie wenn der Zutritt in die Lipoidschicht durch eine in bezug auf die Molekülgrösse selektive Siebplatte zum Teil abgesperrt wäre. Dies weicht von der Vorstellung ab, die den Forschern im allgemeinen vorgeschwebt zu haben scheint, welche den physikalischen Ursachen der Siebwirkung nachgegangen sind, nämlich, dass dem Molekül alternativ zwei nebeneinander einherlaufende Wege zur Verfügung ständen.

Hätte das Molekül tatsächlich zwischen zwei alternativen Wegen, dem des AuflöSENS ins Lipoid und dem Porenwege zu wählen, so wäre

$$P = L + S \quad (3)$$

d.h., die Permeabilität der Zelle wäre gleich der Summe der »Lipoidpermeabilität« und der »Porenpermeabilität«. Innerhalb der Alkoholreihe würde dies nicht so viel Unterschied machen, es wäre aber die im Moment 2 erwähnte Ähnlichkeit der Siebwirkung in bezug auf verschiedene homologe Reihen schwer zu erklären. Die schwächer lipoidlöslichen Amide bedienten sich relativ mehr des Porenweges als die Alkohole, und überhaupt würde die Siebwirkung am deutlichsten bezüglich der am aller schwächsten lipoidlöslichen Stoffe in Erscheinung treten und nicht, wie es sich in Wirklichkeit zu verhalten scheint, in gänzlicher Unabhängigkeit von der Lipoidlöslichkeit.

Zu alledem steht uns auch ein positiver Beweis zugunsten dieser Behauptung zur Verfügung: die Diatomeen, deren Permeabilitätseigenschaften sich als gerade derart herausstellen, wie es die Formel (3) erwarten lässt. In Abb. 1 vertritt Spirogyra den gewöhnlichen »Lipoidtyp«, der für die ver-

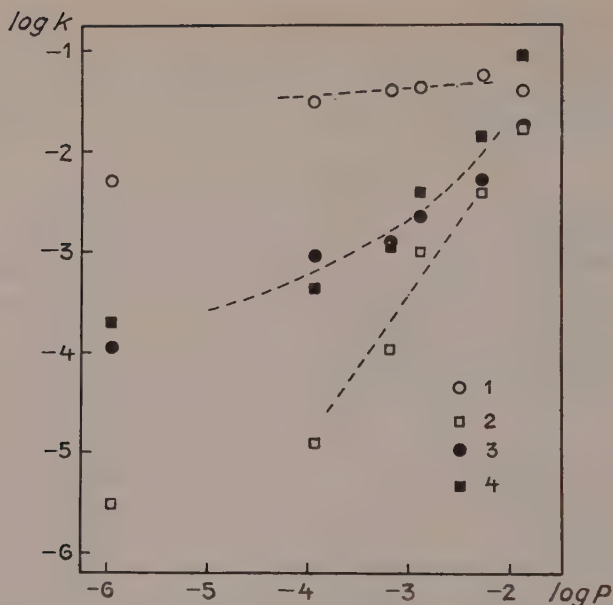


Abb. 1. Permeabilität pflanzlicher Zellen verschiedenen Typs. P = Permeabilität cm/st, k = Verteilungskoeffizient Äthyläther/Wasser. 1 Beggiatoa, 2 Spirogyra, 3 Spirogyra 0,97 + Beggiatoa 0,03, 4 Melosira. Von rechts nach links: Saccharose, Erythritol, Glycerol, Methylharnstoff, Äthylenglykol, Propionamid. (1, 2 und 4 nach Marklund 1936.)

schiedenen Stoffe annähernd im Verhältnis zu ihrer Lipoidlöslichkeit durchlässig ist. Beggiatoa ist als Repräsentant des reinsten bekannten »Porentyps« gewählt worden. Durch Multiplizieren der Permeationskonstante von Spirogyra mit 0,97 und die von Beggiatoa mit 0,03 und paarweise Addition der Produkte sind Permeationskonstanten einer hypothetischen Zelle berechnet worden, deren Plasmahaut grossenteils ähnlich wie bei Spirogyra beschaffen ist, zu 3 % aber die Eigenschaften der Oberflächenschichten der Beggiatoazelle besitzt. Es besteht eine unverkennbare Ähnlichkeit zwischen den Permeabilitätseigenschaften der »synthetischen« Zelle und denjenigen der dritten Pflanze Melosira, die den Diatomeen zugehört.

Bei Nitella äussert sich die Wirkung der zwei Faktoren derart, als ob sie in zwei aufeinanderliegenden Schichten lokalisiert seien. Für zwei nacheinanderfolgende Diffusionswiderstände gilt aber analog mit dem Ohmschen Gesetz

$$\frac{1}{P} = \frac{1}{L} + \frac{1}{S} \quad (4)$$

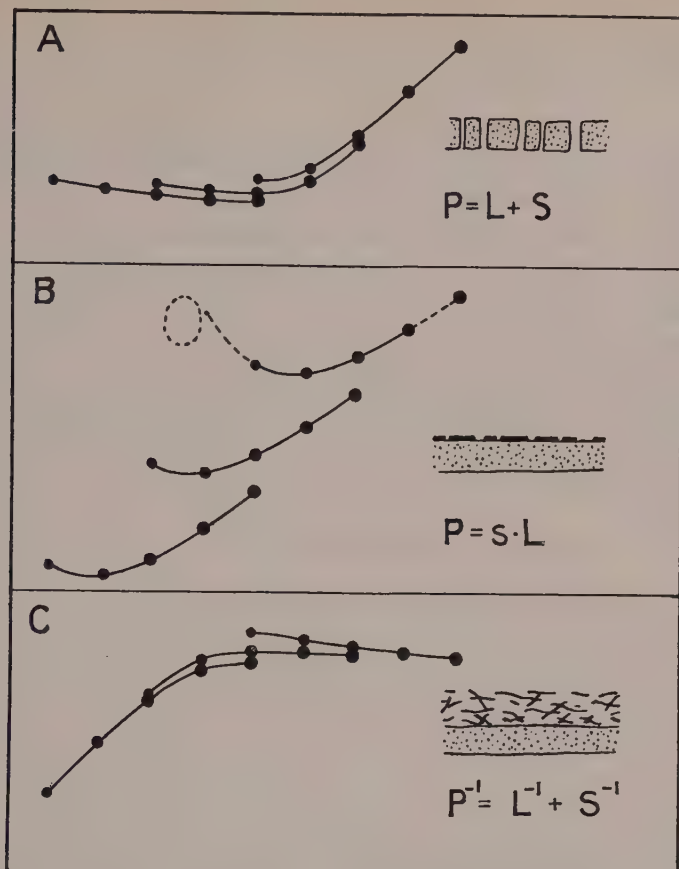


Abb. 2. Einfluss verschiedener Porenstrukturen auf die Permeabilität; schematisch. Abszisse: Verteilungskoeffizient, Ordinate: Permeabilität. Drei der Alkoholreihe ähnliche, nur in bezug auf die Lipoidlöslichkeit verschiedene homologe Reihen. A die Lipoidschicht ist porig. B die Oberfläche ist von einer Lochplatte bedeckt. Oberste Kurve: die Alkohole, der ungenau bekannte Punkt für das Wasser durch Umrandung angedeutet. C Ultrafilter und Lipoidschicht aufeinander gelagert.

Folglich kann das Molekülsieb nicht z.B. ein mit der Zellwand vergleichbarer Diffusionswiderstand sein. Es muss vielmehr gleich einer der Lipoidschicht anliegenden äusserst dünnen Lochplatte wirken, deren effektives Porenareal dem Faktor s proportional ist. Eine solche Platte würde viele kleine und wenige grössere oder elastisch dehnbare Poren haben. Der Diffusionsweg in den Poren wäre verschwindend kurz.

In Abb. 2 ist die Wirkungsweise der besprochenen Porenstrukturen anschaulich zusammengefasst.

Die oberste Kurve in B zeigt die Alkoholpermeabilität von Nitella, auf die Olivenöllöslichkeit bezogen. Die empirischen Zahlenwerte sind nur etwas abgerundet, um für die Faktoren L und s einfache Gleichungen zu bekommen, die bei der Berechnung der anderen Kurven und beim Extrapolieren benutzt wurden ($L = a \cdot n \cdot \sqrt[3]{100}$, $s = \Sigma n^2$, a = eine beliebige Konstante, n = Anzahl der Kohlenstoffatome). Bezüglich der Porenpermeabilität S wurde angenommen, dass sie etwas steiler als $1/\sqrt{M}$ abnimmt.

Möglicherweise könnte an ein aus den polaren Endgruppen der Lipoidmoleküle gebildetes Molekülsieb gedacht werden, dessen Durchlässigkeit bei zunehmender Grösse der permeierenden Moleküle sehr schnell abnimmt. Es spricht aber vieles zugunsten der Auffassung, dass es sich überhaupt nicht um eine wirkliche Siebstruktur, sondern um eine Oberflächenerscheinung handelt (Wartiovaara 1949). Die Plasmahaut dürfte also im wesentlichen homogen sein.

Setzt die Siebwirkung Poren voraus?

Die Moleküle eines schwach lipoidlöslichen Stoffes treten aus der Plasmahaut rasch auf deren Innenseite aus. Die Geschwindigkeit der Permeation hängt mithin praktisch von denjenigen Erscheinungen ab, die sich in der äusseren Grenzfläche abspielen. Unter diesen Erscheinungen müsste auch der Urheber der Siebwirkung zu suchen sein.

Indem sich nun die Siebwirkung anscheinend als eine dermassen generelle Erscheinung herausstellt, dass man ihr in sehr verschiedenen Zellen und in bezug auf verschiedene homologe Reihen gleichartig begegnet, so dürfte sie im Bau der Plasmahaut nur irgendeinen solchen Zug voraussetzen, der für fast sämtliche Zellen gemeinsam ist. Dieser gemeinsame Zug ist nach allem zu schliessen der, dass die hydrophoben Teile der Lipoidmoleküle in ihrer senkrechten Orientierung zur Grenzfläche zu einer einheitlichen Schicht vom Charakter eines Kohlenwasserstoffs zusammentreten: Die chemische Beschaffenheit der Lipoidmoleküle und die Mengenverhältnisse der verschiedenen Lipide variieren dabei sicherlich, desgleichen die Art und Menge des Proteins, der Ionen u.dgl. in der Plasmahaut vorhandenen Substanzen.

Schalten wir nun alles weniger Wesentliche, wie die innere Grenzfläche, die Uneinheitlichkeit der Lipide und etwaige adsorbierte Schichten aus, so gestaltet sich das Modell der Plasmahaut sehr einfach. Man hat da nur die Grenze zwischen zwei Phasen: auf der einen Seite Wasser, auf der anderen senkrecht gegen die Grenzfläche eingestellte Paraffinketten, deren äusserstes Ende wir uns vielleicht hydrophil denken müssen.

Es gilt nun zu untersuchen, ob eine solche Grenzfläche die Fähigkeit besitzen kann, in der Art eines Molekülsiebes auf die diffundierenden Mole-

küle der niederen Alkohole einzuwirken, und, falls dies theoretisch möglich erscheint, ob diese Siebwirkung derjenigen des lebenden Protoplasmas ähnlich sein kann.

Da die Gesetze betreffs der Diffusion und Osmose in Flüssigkeiten den entsprechenden Gasgesetzen weitgehend analog sind, untersuchen wir, ob sich vielleicht auch die Siebwirkung gaskinetisch beschreiben liesse. Die Theorie des flüssigen Zustandes ist bekanntlich verwickelt, was die Benutzung dieses Umweges berechtigen dürfte.

Die Alkoholmoleküle denken wir uns in Form von steifen Stäbchen, etwa wie gekürzte Zündhölzchen. In einem Gas weicht zwar die durchschnittliche Form der Moleküle wegen der relativ unbehinderten intramolekularen Rotation der Kohlenstoffatome erheblich hiervon ab, in einer Flüssigkeit dürfte aber die Kohlenstoffkette gerader sein (vgl. Staudinger, Bier und Lorentz 1949). Auch wenn wir das Wasser als Medium unberücksichtigt lassen, behalten wir die Alkoholmoleküle in derselben Form bei, wie wir sie uns in einer Wasserlösung vorstellen. Es gilt ja hier nicht die Diffusion des wirklichen Alkoholdampfes in das Lipoid zu untersuchen, sondern eine eventuelle Analogie zwischen der Permeation und deren gaskinetischem Modell zu ermitteln.

In einem idealen Gas sind die Bewegungen der Moleküle völlig zufallsbedingt. Sie treffen auf die Lipidoberfläche aus allen möglichen Richtungen und mit verschiedener Geschwindigkeit auf. Die Ankunftsrichtung und die mittlere Energie der translatorischen Bewegung sind unabhängig von den individuellen Eigenschaften der Moleküle. Diese Faktoren äussern sich also, einerlei um welches Glied der Alkoholreihe es sich auch handeln mag, stets gleichartig und brauchen hier darum nicht weiter beachtet zu werden.

Wir wählen zum Gegenstand unserer Betrachtung ein Molekül, das sich direkt gegen die Grenzfläche bewegt und so rotiert, dass es abwechselnd das Hydroxyl und die Methylgruppe der Richtung der translatorischen Bewegung zukehrt, dass also zufälligerweise die Rotationsebene die Grenzfläche rechtwinklig schneidet. Die Paraffinketten der Lipoidphase befinden sich ebenfalls in unablässiger Bewegung. Sie können einzeln und in Gruppen in mannigfacher Weise Schwingungen ausführen, so dass man an der Oberfläche vielleicht eine stark bewegte »Brandung« erblicken würde, die kleine, sich alsbald wieder schliessende Lücken zwischen die Paraffinketten einreisst. Vgl. Abb. 3.

Was geschieht nun, wenn das Alkoholmolekül die Lipidoberfläche trifft? In der Mehrzahl der Fälle prallt es zurück, denn bei gewöhnlicher Temperatur besitzen nur wenige Moleküle die zur Überwindung des an der Grenzfläche herrschenden Widerstandes erforderliche Energiemenge. Dieser Widerstand wird vornehmlich von der Wasserstoffbindung zwischen Hydroxyl und Wasser gebildet, die aufgehoben werden muss. Betreffs der genaueren Einzelheiten möge auf die Berechnungen Daniellis (Davson und Danielli 1943) verwiesen werden. Für uns genügt die Feststellung, dass die kleinste Energiemenge, die das Molekül haben muss, damit es aus dem Wasser ins Lipoid übertreten kann, annähernd proportional dem Verteilungskoeffizient ist.

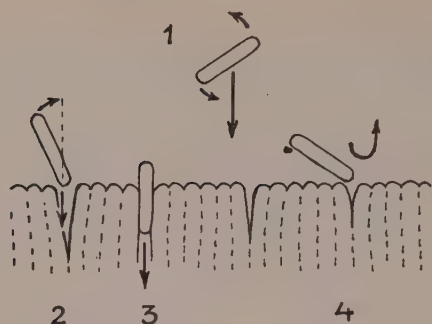


Abb. 3. Das Eindringen von stäbchenförmigen Molekülen in die aus parallel gestellten Paraffinketten bestehende Plasmahaut (grob schematisch). 1. Im Wasser frei herumwirbelndes Molekül. — 2. Mit dem einen Ende anstossendes Molekül, das zwischen die Plasmahautmoleküle eindringen wird. — 3. Molekül halbwegs in die Lipidschicht eingedrungen. — 4. Allzu schief anstossendes Molekül, das von der Plasmahaut zurückprallt.

Verfügt nun das Molekül im Augenblick des Auftreffens zufälligerweise über eine Energiemenge, die den eben erwähnten absoluten Mindestbetrag übertrifft, so bedeutet dies immer noch nicht, dass es ihm sicher gelingt, die Grenzfläche zu durchdringen. Es muss für das Molekül in der Lipidschicht Raum bereitet werden. Derart grosse Geschwindigkeiten, dass das Molekül in der Art eines Projektils alle Hindernisse zur Seite stiesse, kommen bei gewöhnlicher Temperatur nicht in Frage. Das Vorkommen kleiner Löcher im Lipoid ist wahrscheinlicher als dasjenige langer Spalte, in die das Molekül auch in seitlicher Lage einzudringen vermöchte. Befindet sich also beim Auftreffpunkt eine Lücke oder wird eine solche auf Kosten der von dem Molekül mitgebrachten Energie gebildet, so dürfte sie praktisch genommen immer so klein sein, dass das Molekül nur mit dem Ende voran anprallend in ihr stecken bleiben kann. Diffundiert es in die Lipoidphase hinein, so wird die senkrechte Lage vermutlich dauernd beibehalten, weil die dichte Packung der gleichsinnig orientierten Lipoidmoleküle die Rotation stark beeinträchtigt.

Es muss also ausser den eben besprochenen Bedingungen der Permeation auch die räumliche Lage des Moleküls berücksichtigt werden. Da der Nettobetrag der Energieumsetzungen beim Übertritt aus der einen Phase in die andere unabhängig davon ist, welches von den beiden Enden vorangeht, so dürften wir der Einfachheit halber annehmen können, dass das Hydroxyl gegen die Lipoidphase zugewendet sein muss, während die Kohlenstoffkette rechtwinklig von ihr abragt.

In unserem gedachten Fall, wo die Rotationsebene mit der Grenzfläche einen rechten Winkel bildet, wird das Molekül einmal während jeder

Umdrehung die genannte Lage durchlaufen. Wenn dies gerade im Augenblick des Zusammentreffens mit der Lipidoberfläche stattfinden müsste, um das Eindringen des Moleküls zu ermöglichen, so würde es bezüglich der Permeabilität keinen Belang haben. Lange Moleküle rotieren freilich träger als kurze, befinden sich aber dafür länger in jeder Stellung. Ganz anders gestaltet sich die Sache, wenn das Molekül noch nachdem es mit der Lipidschicht zusammengetroffen ist, sich senkrecht einstellen kann. Es hängt dann von der Grösse seines Trägheitsmoments ab, ob diese Orientierung Zeit findet, sich schon vor dem Zurückprallen zu vollziehen, und wieviel hierbei die ursprüngliche Lage von der senkrechten abweichen darf. Dass sich die zwei Teilvorgänge der Permeation, das Einbrechen ins Lipoid und die Orientierung, recht wohl gleichzeitig abspielen könnten, erhellt daraus, dass die Orientierung jedenfalls in einem flüssigen Medium als eine Diffusionserscheinung aufzufassen ist, deren Geschwindigkeit mit der translatorischen Geschwindigkeit grössenordnungsgemäss übereinstimmen dürfte.

Das Trägheitsmoment lässt sich ziemlich genau berechnen (vgl. z.B. Hougen und Watson 1948). Für vorliegende Zwecke genügt uns eine einfache Schätzung. Wir stellen uns das Molekül als einen steifen Stab vor, dessen Masse gleichmässig auf mehrere in gleichen Abständen voneinander gelegene Punkte (die OH-, CH₂-, und CH₃-Gruppen vertreten) konzentriert ist. Wir stellen uns ferner das Molekül so verankert vor, dass es sich um den Hydroxyl-Massenpunkt frei wenden kann. Als Längeneinheit wählen wir am bequemsten den Abstand zwischen zwei aufeinanderfolgenden Massenpunkten und als Einheit für die Masse diejenige eines solchen Punktes. Das Trägheitsmoment in bezug auf eine beliebige Drehungsachse ist

$$I = \Sigma m \cdot d^2 \quad (5)$$

wenn m die Masse eines Massenpunktes und d seinen Abstand von der Achse bedeuten. Weil nun die Achse durch das Hydroxyl geht, wird d alle ganzzahligen Werte von 0 bis n annehmen, wenn das Molekül n Kohlenstoffatome enthält. Da ferner $m = 1$ gesetzt wurde, so vereinfacht sich die Gleichung auf

$$I = \Sigma(n) = 1 + 4 + 9 + \dots + n^2. \quad (6)$$

Je kleiner das Trägheitsmoment ist, desto weniger wird die Wahrscheinlichkeit der Permeation durch die Zufallsbedingtheit der Molekülstellung beeinträchtigt. Die Siebwirkung müsste also dem Trägheitsmoment umgekehrt proportional sein. Wie es Tab. 2 zeigt, ist dies in betreff der Alkoholpermeabilität von Nitella tatsächlich der Fall, und zwar mit einer Genauigkeit, die als befriedigend anzusehen ist. Ist es doch, von den nur annäherungsweise richtigen Werten des Trägheitsmoments und von unvermeidlichen Versuchsfehlern abgesehen, u.a. nicht erwiesen worden, dass der Verteilungskoeffizient im System Olivenöl-Wasser als Ausgangspunkt für die Berechnung der Siebwirkung theoretisch ganz einwandfrei ist.

Es möge bemerkt werden, dass sich das Wasser ausgezeichnet zur Fort-

Tabelle 2. Siebwirkung (s) und Trägheitsmoment (I).
Relative Werte, Methanol=100.

	s	$1/I$
Wasser	(1000)	(1000)
Methanol	100	100
Ethanol	18	20
Propanol	7.4	7.2
Butanol	3.8	3.3

setzung der Alkoholreihe eignete. Hierzu könnte vielleicht eingewendet werden, dass es ja beim Wasser kaum etwas zu richten gäbe. Ist doch da nicht einmal eine solche rudimentäre »Kette« wie beim Methanol vorhanden. In Wirklichkeit ist die Gegensätzlichkeit der beiden Enden des Wasser-Dipols schroffer als die beim Methanol zwischen dem Hydroxyl und der Methylgruppe herrschende. Es dürfte wahrscheinlich sein, dass auch das Wassermolekül die Lipidoberfläche in ganz bestimmter Stellung am leichtesten durchdringt oder dass mit dem Zerreißen der Wasserstoffbindung stets auch eine volle Umkehrung des Dipols verbunden ist. Die Lipidlöslichkeit und das Trägheitsmoment des Wassers sind beide etwa um eine Zehnerpotenz kleiner als die des Methanols, was gut mit der Tatsache im Einklang steht, dass die Permeationsgeschwindigkeit dieser beiden Stoffe in die gleiche Größenordnung fällt. Beim Wasser wird der permeationshemmende Einfluss der kleineren Lipidlöslichkeit durch das entsprechend kleinere Trägheitsmoment aufgehoben. (Vgl. Collander 1949.)

Die wirkliche Permeation unterscheidet sich von unserem gedachten Fall in der Beziehung, dass ihre sämtlichen Teilerscheinungen diskontinuierlich sind. Das Molekül schwingt tausende Male um seine Gleichgewichtslage herum, ehe es im Verhältnis zu den Nachbarmolekülen eine neue Gleichgewichtslage einnimmt. Es kann sich nicht mit derselben Wucht der Grenzfläche sowohl nähern als sie auch durchdringen, sondern mag mehrmals während seines Weges Energie empfangen und abgeben. Das einzelne Molekül benimmt sich also, im einzelnen verfolgt, in einer Flüssigkeit ganz anders als in einem Gas. Dessenungeachtet schreiten die Diffusionsvorgänge, im grossen betrachtet, in einer Flüssigkeit freilich träger, sonst aber ähnlich wie in einem Gas fort. Es besteht kein Grund anzunehmen, dass die zur Erklärung der Siebwirkung aufgeworfene Orientierung in dieser Hinsicht eine Ausnahme repräsentierte. Sie vollzieht sich diskontinuierlich, auf dem Wege einer Art Diffusion, mit einer Geschwindigkeit, die dem Trägheitsmoment umgekehrt proportional ist.

Es sei ausdrücklich betont, dass das Vorangehende keineswegs als eine erschöpfende Beschreibung der tatsächlichen Bewegungen der permeierenden

Moleküle aufzufassen ist. Es handelt sich vielmehr um eine Arbeitshypothese, die sich betreffs des leitenden Gedankens wohl auf der richtigen Spur bewegen mag, die aber nicht streng wörtlich genommen werden darf. Eine beim Übertritt in die Lipoidphase stattfindende Entknäuelung der Kohlenstoffkette würde z.B. eine gleichartige Wirkung haben wie die Orientierung eines starren Moleküls, ebenso wie jede durch die Anisotropie und die dichte Packung der Plasmahautlipoide bedingte Einschränkung der Freiheitsgrade bezüglich Form und Bewegung, aus thermodynamischen Gründen, zu der Siebwirkung beitragen muss.

Wie sich die Einzelheiten auch herausstellen werden — das letzte Wort in dieser Sache gehört natürlich dem Physiker — auf jeden Fall lässt sich behaupten, dass *sich die Siebwirkung auch ohne die Annahme jedweder Porenstruktur ungezwungen erklären lässt. Nicht einmal die hohe Permeabilität für Wasser setzt Poren voraus.*

Vergleichende Betrachtungen

Die träge Orientierung der langen Moleküle an der Oberfläche der Plasmahaut scheint vielleicht in Widerspruch mit ihrer bekanntlich grossen Oberflächenaktivität zu stehen. Dieser Widerspruch ist aber nur scheinbar. Die Traubesche Regel betrifft ja den Gleichgewichtszustand, nicht die Geschwindigkeit der Orientierung. Es sei an den Unterschied zwischen der statischen und dynamischen Oberflächenspannung erinnert, der zumal bei den höheren Homologen ausgeprägt hervortritt.

Wird die Siebwirkung auf Orientierungsvorgänge zurückgeführt, so müssten auch unbelebte Grenzschichten von dicht aneinanderschliessenden orientierten Lipoidmolekülen eine ausgesprochene Siebwirkung zeigen können. Nach Hutchinson (1948) vermag an die Grenzfläche zwischen Wasser und Benzen adsorbiertes Cetylsulphat die Diffusion von niederen Alkoholen auch tatsächlich zu verzögern, und zwar wird Butanol entschieden stärker als Ethanol in der wässerigen Phase zurückgehalten. Die Übereinstimmung mit der Siebwirkung des Protoplasmas von *Nitella* ist freilich nur qualitativ, es liegt aber die Vermutung nahe, dass in beiden Fällen derselbe physikalische Faktor tätig ist.

Einen besonders schwerwiegenden Beweis zugunsten der hier besprochenen Auffassung stellen die Ergebnisse von Äyräpää (1950) über die Permeabilität von Hefezellen für aliphatische Amine dar. Der physiologischen Verschiedenheit des Objekts und dem ganz andersartigen chemischen Charakter der permeierenden Moleküle zum Trotz, besteht zwischen der Siebwirkung und dem Trägheitsmoment eine ähnliche Korrelation wie sie für *Nitella* be-

züglich der primären Alkohole gefunden wurde. Man bemerke, dass die Dimensionen und Symmetrieverhältnisse der Moleküle primärer Alkohole mit denjenigen der entsprechenden Aminmoleküle bis auf einen geringen Unterschied in der hydrophilen Endgruppe übereinstimmen.

In den Alkohol- und Aminreihen bildet die kleine Endgruppe gewissermassen eine unmittelbare Fortsetzung der Kohlenstoffkette, so dass die Moleküle als Stäbe mit einem ausgeprägten Hauptträgheitsmoment aufgefasst werden können. Anders verhält es sich bei den als Plasmolytika beliebten mehr hydrophilen Verbindungen. Der Regel nach besteht die hydrophile Gruppe aus mehreren polaren Atomen (Fettsäureamide, Alkylharnstoffe) oder es sind mehrere hydrophile Gruppen vorhanden (Glykole). Da ausserdem die Kohlenstoffkette kurz zu sein pflegt, so werden die Symmetrieverhältnisse verwickelt und der Zusammenhang zwischen der Siebwirkung und der Orientierung weniger durchsichtig. Ja, es mag unter solchen Umständen die Lage des Moleküls tatsächlich an Bedeutung verlieren. Ein kompakt gebautes Molekül kann in mancherlei Weise gedreht werden, ohne dass die entsprechenden Trägheitsmomente entschieden ungleich sind, oder es mögen gar mehrere Lagen hinsichtlich der Permeation gleichwertig sein (Pentaerythrit).

Für wenig bzw. mässig lipoidlösliche Stoffe gilt nach Danielli (Davson und Danielli 1943) die theoretische Gleichung

$$P = \frac{a}{2} = \frac{1}{2} r \Phi_a \sqrt{\frac{RT}{2\pi M}} e^{\frac{\mu_a}{RT}} \quad (7)$$

wo P die Permeabilität, a die Diffusionskonstante bei Diffusion aus Wasser ins Lipoid, r eine Konstante, Φ_a die Wahrscheinlichkeit, dass ein Molekül mit der Energie μ_a ins Lipoid übertritt. R die Gaskonstante, T die absolute Temperatur und M das Molekulargewicht bedeuten. Es ist also die Durchlässigkeit der äusseren Phasengrenze massgebend, die ihrerseits, konstante Temperatur und sonstige Umweltfaktoren angenommen, von Φ_a , μ_a und \sqrt{M} abhängig ist. Wir können die Wirkung des letztgenannten Faktors vernachlässigen, da der Wert von \sqrt{M} für Butanol nur etwa anderthalbmal grösser als für Methanol ist. Es bleiben also zwei mit den Eigenschaften der permeierenden Moleküle sich verändernde Faktoren Φ_a und μ_a zurück, von denen μ_a zu der Lipoidlöslichkeit in Beziehung steht und Φ_a nach Danielli grossenteils ein Orientierungsfaktor ist. Die thermodynamisch abgeleitete Gleichung (7) stimmt demnach mit der empirischen (1) in den wesentlichen Punkten überein, dass beide den Orientierungsfaktor als einen Koeffizienten enthalten und sonst ausser einem Energiefaktor bzw. der von dem Energiefaktor abhängigen Lipoidlöslichkeit keine anderen Variablen einschliessen, falls T und M als konstant angenommen werden.

Die Gleichung (1) basiert sich auf die Behauptung, dass die Existenz der

Siebwirkung eine empirische Tatsache ist, was aber Danielli sehr ernstlich in Abrede stellt. Es dürfte die Übereinstimmung zwischen den Gleichungen nur so zu verstehen sein, dass die Vertreter der gegensätzlichen Anschauungsweisen, Collander und Danielli, in Wirklichkeit beide recht haben: Collander darin, dass die Siebwirkung tatsächlich existiert und gewissermassen mit den Dimensionen der Moleküle — jedenfalls mit ihrer Länge — in Beziehung steht, Danielli seinerseits darin, dass die Plasmahaut der Regel nach wesentlich homogen ist, also keine Siebstruktur besitzt, und dass das Molekularvolumen an sich die Permeation nicht nachweisbar beeinflusst.

Zusammenfassung

Die physikalischen Ursachen der Ultrafilterwirkung des Protoplasmas werden auf Grund von Literaturangaben über die Durchlässigkeit der Pflanzenzellen theoretisch besprochen.

Es werden zwei verschiedene Hauptformen der Ultrafilterwirkung unterschieden, je nachdem die gemäss der Lipoidlöslichkeit und gemäss der Molekülgrösse selektiven Faktoren parallel oder nacheinandergeschaltet erscheinen. Die erstere Form, hier Porenpermeabilität genannt, ist selten deutlich ausgeprägt, wie z.B. bei den Diatomeen, und existiert der Regel nach nur gleichzeitig mit der letzteren. Sie beruht wahrscheinlich auf einer ungleichmässigen Durchlässigkeit der Plasmahaut, die mit der physiologischen Differenzierung in Beziehung steht.

Die andere allgemeine Form wird zum Unterschied von der ersteren als Siebwirkung bezeichnet und einer eingehenderen Analyse unterworfen, bei welcher die früheren Ergebnisse des Verfassers über das Permeationsvermögen niederer Alkohole als Ausgangspunkt dienen.

Innerhalb der homologen Reihe ist die Siebwirkung als eine relative Disproportionalität zwischen Lipoidlöslichkeit und Permeationsvermögen zumindest bis zum vierten Glied nachweisbar. Der Diffusionswiderstand des Protoplasmas den einzelnen Gliedern der Reihe gegenüber kann durch das Produkt der Zahlenwerte zweier Faktoren, Lipoidlöslichkeit und Siebwirkung, ausgedrückt werden, wie wenn in der Plasmahaut ein Molekülsieb und eine lückenlose Lipoidschicht aufeinanderengelagert seien.

Dem Lipoidlöslichkeitsfaktor wird die übliche Deutung gegeben, betreffs der Siebwirkung aber der Hypothese des Verfassers gemäss angenommen, dass es sich um einen von der Länge und der zufälligen räumlichen Lage des permeierenden Moleküls abhängigen Orientierungsfaktor handele. Es wird vermutet, dass die von dicht gepackten orientierten Lipoidmolekülen gebildete Plasmahaut die als starre Stäbchen angenommenen Alkoholmoleküle vorzugsweise in senkrechter Stellung zu ihr durchlässt. Die Lage eines

gegen die Phasengrenze anprallenden Moleküls wird Hand in Hand mit der zunehmenden Länge seiner Kohlenstoffkette mehr kritisch und seine nachträgliche Orientierung schwieriger. Die durch die Lipoidlöslichkeit bestimmte maximale Anzahl effektiver Zusammenstöße wird also bis auf einen, von der Gestalt der Moleküle abhängigen Bruchteil herabgesetzt.

Zugunsten dieser Hypothese sprechen zumal folgende Umstände:

1. Die den Siebhypothesen eigenen Widersprüche lassen sich vermeiden. Der offenbar allgemeinen Natur der Siebwirkung entsprechend, braucht man der Plasmahaut nur die, gleichfalls allgemeine und so gut wie sichergestellte Eigenschaft zuzuschreiben, wesentlich von senkrecht gerichteten Lipoidmolekülen aufgebaut zu sein.

2. Es besteht eine Korrelation zwischen dem Trägheitsmoment der betreffenden Moleküle und der Siebwirkung.

3. Jene Korrelation erstreckt sich grössenordnungsgemäss bis zum Wasser. Es ist also nicht notwendig, einen ganz speziellen Permeationsmechanismus für das Wasser anzunehmen.

Die Hypothese erscheint physikalisch plausibel und wird von einer der neuesten Untersuchungen auf dem Gebiete der Permeabilitätsforschung gestützt.

Zitierte Literatur

- Äyräpää, T.: On the base permeability of yeast. — *Physiol. Plant.* 3: 402. 1950.
- Collander, R.: The permeability of plant protoplasts to small molecules. — *Physiol. Plant.* 2: 300. 1949.
- Die Verteilung organischer Verbindungen zwischen Äther und Wasser. — *Acta Chem. Scand.* 3: 717. 1949.
- & Bärlund, H.: Permeabilitätsstudien an *Chara ceratophylla*. II. — *Acta Bot. Fenn.* 11: 1. 1933.
- & Wikström, B.: Die Permeabilität pflanzlicher Protoplasten für Harnstoff und Alkylharnstoffe. — *Physiol. Plant.* 2: 235. 1949.
- Davies, J. T.: The mechanism of diffusion of ions across a phase boundary and through cell walls. — *J. Phys. & Colloid Chem.* 54: 185. 1950.
- Davson, H. & Danielli, J. F.: The permeability of natural membranes. — Cambridge 1943.
- Drawert, H.: Zellmorphologische und zellphysiologische Studien an Cyanophyceen. I. — *Planta* 37: 161. 1949.
- Hougen, O. A. & Watson, K. M.: Chemical process principles. II. — New York, London 1948.
- Hutchinson, E.: Diffusion across oil-water interfaces. — *J. Phys. & Colloid Chem.* 52: 897. 1948.
- Höfler, K.: Aus der Protoplasmatik der Diatomeen. — *Ber. D. Bot. Ges.* 58: 97. 1940.
- Staudinger, H., Bier, G. & Lorenz, G.: Viskositätsuntersuchungen an niedermolekularen Estern mit verzweigten Molekülen. — *Makromol. Chem.* 3: 251. 1949.
- Wartiovaara, V.: The permeability of *Tolypellopsis* cells for heavy water and methyl alcohol. — *Acta Bot. Fenn.* 34: 1. 1944.
- The permeability of the plasma membranes of *Nitella* to normal primary alcohols at low and intermediate temperatures. — *Physiol. Plant.* 2: 184. 1949.

Stomatal Behaviour in Buffer Solutions

By

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I. Introduction

Apart from the preliminary experiments of Sayre (8), which being purely exploratory may be excluded from the criticism, work on stomatal behaviour in buffer solutions has been surprisingly naïve. Small *et al.* (10, 11), Saïd and Tolba (7), and Alvim (1) have all carried out investigations involving the immersion of epidermal strips in buffer solutions (usually M/10 or M/20) and have noted movements: all have assumed that these movements are the result of the pH of the solution, and not one seems to have considered the possibility that the mere presence of salts in relatively high concentrations might itself have a marked effect: and in fact not one has referred to the exhaustive investigations of Arends (2) into this subject. This criticism has already been voiced by Heath (5): »As already noted, by immersing detached epidermis in a variety of buffer mixtures Small *et al.* (*loc. cit.*) obtained violent fluctuations in both stomatal aperture and starch content with pH . . . in view of the complex effects of salts on stomata in detached epidermis found by Arends (1926) and others, this is perhaps not surprising».

We recently initiated a further investigation with this possibility in view. Unfortunately, after only a short time the work has had to be adjourned *sine die* owing to the departure of the junior author to take up other commitments; but some of the results obtained seem to us to be of sufficient interest to justify this brief report.

II. Experimental Method

We have confined our experiments to *Pelargonium zonale* var. 'Paul Crampel'. Although we have in general adhered to the methods of earlier

workers, we have controlled one further factor which they do not mention: the illumination — or otherwise — of the strips while in the buffer solutions. It is clear from the work of Arends (2) that a light effect on salt-induced movements may be expected (we are indebted to Professor L. Brauner for drawing our attention to this phenomenon), and the possibility of such an effect has prevented us from following the course of movement of individual stomata. Our procedure has therefore been as follows: — Strips of epidermis, taken from the underside near the outer margin of the same leaf, were all put into the test solution at the same time; half the strips were in dishes under a bank of daylight fluorescent lamps giving an intensity of approximately 750 candle-power, the others were also put under the lamps (to minimize temperature differences) but covered over. The temperature difference between the two sets did not exceed 2°C , and, since Alvim (1) found that quite large temperature changes did not affect buffer-induced movements, we consider that differences in the two sets may reasonably be attributed to light effects. Strips were withdrawn at half-hour intervals and the width of 20 stomata measured, using a $\frac{1}{7}$ -inch dry objective and a $\times 18$ eyepiece with micrometer scale; one unit of the latter was approximately equal to $2\ \mu$. The pH of all solutions used was measured electrometrically.

III. Results

1. *Acetate solutions at c. pH 7*

Alvim reports that maximum opening of *Pelargonium* stomata in acetate buffers occurs at pH 6.9—7.3. Now, the sodium-acetate/acetic-acid system buffers over only a narrow range; and a glance at a graph of »buffering capacity» against pH for this mixture, such as is given in Glasstone (3, p. 214), will immediately show that the buffering capacity has already fallen to about a quarter of its maximum value by pH 6, and reaches zero at about pH 7.1. In fact, in the range pH 6.9—7.3 Alvim was virtually dealing with a solution of sodium acetate, whose buffering powers would be negligible until the pH fell to about 6. We found that a M/10 solution of sodium acetate in glass-distilled water (which probably contained a little dissolved CO_2) had already a pH of 7.13, and this solution, without admixture of acetic acid, was used in the following experiments. We agree with Alvim that Small's preliminary washing in »neutral water» had no discernible effect, and its use was therefore abandoned.

The results of one experiment are given in the first three columns of Table 1. It is immediately clear that there is (a) an opening effect in this solution, and (b) a marked light effect, the illuminated stomata opening about

Table 1.

Hours	Mean aperture, units				Starch status in Expt. 2 (light)		
	Expt. 1		Expt. 2				
	Light	Dark	Light	Dark	Full	Half	Empty
0	0.275	0.20	0.65	—	100	0	0
1/2	0.85	0.25	2.95	0.40	—	—	—
1	0.65	0.375	2.05	0.65	—	—	—
1 1/2	0.725	0.725	2.25	0.85	—	—	—
2	0.85	0.50	2.75	1.10	92.7	5.7	1.6
2 1/2	1.025	0.475	2.95	1.05	81.3	11.8	6.9
3	0.90	0.45	(1.70)	(0.70)	75.9	18.5	5.6
3 1/2	0.925	0.475	(1.65)	(0.65)	73.4	11.9	14.7
4	—	—	(1.45)	(0.00)	81.6	15.8	2.6

twice as widely as those in the dark. The results of a replicate experiment are given in the fourth and fifth columns of the same table (excluding the values in brackets): the effect here is even more striking, and incidentally illustrates the wide variation in behaviour that may occur between different leaves (in this case from different plants). After 2 1/2 hours the conditions in this experiment were reversed, the illuminated dishes being darkened and *vice versa*. The results, which are shown in the same columns in brackets, are surprising: there is a marked closing effect in both. Evidently the light effect differs from normal light-induced movements in that it is not simply reversible.

The opening effect in Expt. 2 is so marked that it should provide a good test of the hypothesis that »buffer» openings are due to starch-sugar or similar changes. We had expected a diminution of starch as opening proceeded, though examination of column 4 of Table 1 shows that there was no significant further opening after the first half-hour; and we had planned to make a quantitative estimate of starch-content by means of the method of Williams and Spencer (13). The strips were therefore plunged into absolute alcohol after measurement — a process unlikely to affect the starch-content — and examined at leisure in Heath's reagent (Heath, 4). Preliminary examination of one of the later strips showed a most unexpected state of affairs: the stomata, instead of showing a reduced starch-content, had either both guard-cells full of starch (Fig. 1 a), one guard-cell full and the other practically empty (Fig. 1 b), or both practically empty (Fig. 1 c). In other words, starch disappears discretely, guard-cell by guard-cell; and it must do so rapidly, since practically no intermediate states were found — cells had either abundant starch or almost none. This is presumably the explanation of Alvim's (1) statement for *Pelargonium*: »*Geranium* also showed a large number of stomata *without* starch when opening was induced by buffers, and

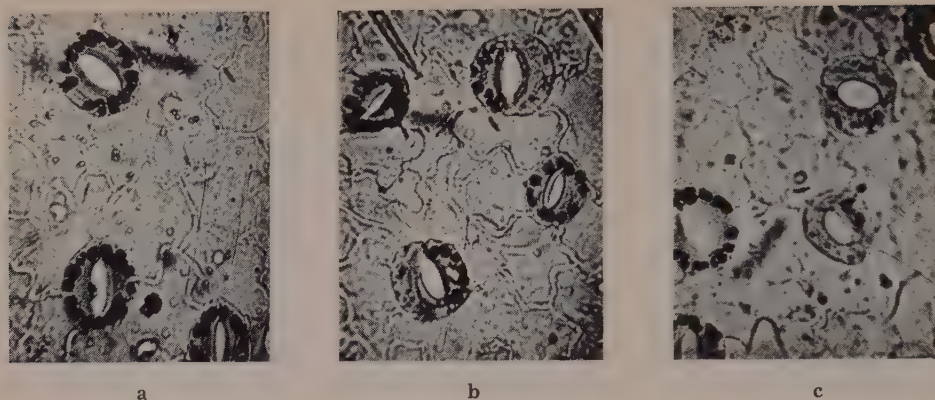


Fig. 1. *Disappearance of starch from individual guard-cells in sodium acetate solution.*

a striking *reduction* in the starch content, when induced by light». (Our *italics*.)

We therefore estimated starch by *counting* (a) the number of stomata with both guard-cells containing abundant starch, (b) the number with one empty guard-cell, and (c) the number with both cells empty. The resulting figures, based on not less than 200 stomata for each strip and reduced to percentages, are given in the last columns of Table 1 under the headings, respectively, of »full», »half», and »empty». Disappearance was not observed until after 2 hours' immersion, and is clearly not related to the opening movement, which is virtually complete long before; nor is there any evidence of a significant reversal of the process corresponding to the closure on later darkening. The phenomenon was not shown by the darkened strips; and in Expt. 1 it was only beginning in the last of the illuminated strips (with 92 % — 6.8 % — 1.2 %). It does not follow, of course, that there is not also a gradual diminution of starch which genuinely controls the opening; but if there is it is too small to be detected by simple inspection.

2. *Other approximately neutral solutions*

The sodium acetate result was only to be expected. The results of Arends, and those of Steinberger quoted by him, show that in general the stomata open when epidermal strips are immersed in solutions of a wide range of neutral salts, provided that the ions concerned are able to enter the cells; this is probably always the case with monovalent cations such as Na and K. We therefore carried out a similar experiment using M/10 KCl, a solution with no buffering capacity whatsoever.

Table 2.

Hours	KCl		Phosphate buffer	
	Light	Dark	Light	Dark
0	1.025	0.90	0.775	0.25
1/2	0.95	0.80	0.85	0.40
1	1.225	1.325	0.85	0.40
1 1/2	1.75	1.675	0.80	0.35
2	1.95	1.675	0.75	0.325
2 1/2	2.10	1.40	0.625	0.125
3	2.20	1.55	0.35	0.10
3 1/2	—	—	0.425	0.10

The results are shown in the first three columns of Table 2, in which the opening effect is clear. The light effect is less well-marked, but appears to be established within about two hours; we could discern no convincing starch-changes. This result cannot possibly be attributed to buffer effects.

We also tried a M/10 KOH-KH₂PO₄ buffer solution at pH 7.25, the results of which are shown in the remaining columns of Table 2. The light effect is clear, but there is if anything a tendency to close. This result, which is not in accordance with those obtained by Small and his colleagues using Sørensen's phosphate buffers, obviously calls for further investigation. It may be due to the strength of our solution; Arends notes that in contrast to their tolerance towards neutral salts, which can safely be used in concentrations of M/2, stomata are rapidly killed by basic salts such as NaHCO₃ and Na₃PO₄ in dilutions exceeding about M/25.

3. Acetate buffers at c. pH 4.6

Alvim forbears to point out one striking discrepancy between his results and those of Small. Small *et al.* (11), using sodium-acetate/acetic-acid buffers, found maximal opening for *Pelargonium* at pH 4.5, the degree of opening falling off on either side of this value. Alvim, using similar buffers, found the stomata closed at all pH values below 6.3, in other words over the whole effective buffered range. We hoped to resolve this discrepancy, and have followed Small's method as closely as possible in an attempt to repeat his result. Admittedly, the pH of the solution we used was 4.66, but according to Small's graph this should still cause marked opening. We have tried M/10 solutions (as used by Alvim) and M/20 solutions (as presumably used by Small, since he states that he used Walpole's mixtures); we have tried washing in »neutral water», as advocated by Small, and omitting to do so, as advocated by Alvim; we have left strips both in light and in darkness, since neither author specifies this condition; and in every case the stomata closed, as found by Alvim. It is possible, of course, that Small had the misfortune

to select a plant abnormal in this particular. Apart from this possibility, we can only suggest that his result was a misinterpretation attributable to the fact that Small and his colleagues (*vide* Small and Maxwell, 10) dispensed with a coverslip to avoid trapping CO_2 (though the importance of this precaution when using strongly buffered solutions is open to question). A relatively low power must have been used, and we have repeatedly found that *Pelargonium* stomata almost without exception entrap air when mounted in aqueous solutions, and that the resulting optical effects can be most misleading unless a relatively high magnification is used.

We repeated the experiment using potassium instead of sodium acetate (pH 4.60). The results were substantially the same for the illuminated strips, but there appeared to be a difference in darkness; in the first experiment »potassium closure» lagged somewhat behind that of the corresponding strips in sodium acetate, and in the replicate this lag was quite marked. We are not satisfied that this is a real effect, but we record it since it suggests that the precise cation selected for the buffer solutions may itself affect the results.

4. *Wide-open stomata*

Arends notes that if epidermal strips of *Zebrina* with wide-open starch-free stomata are immersed in water, the stomata rapidly close and starch appears, a somewhat complex light effect being discernible here also. Wide-open, even relatively starch-free stomata of *Pelargonium* can only be obtained, in this climate at least, by enclosing a leaf in a moist CO_2 -deficient atmosphere, conveniently obtained by enclosing the leaf between two glass plates and illuminating strongly. Strips from leaves so treated do not exhibit stomatal closure or any evident starch-change on immersion in water, either in light or darkness. This is not unexpected, since the senior author (12) has observed in other connexions that a moist atmosphere favours stomatal opening, or at least retards closure, in *Pelargonium*. The difference between the two genera need excite no surprise: as long ago as 1856 von Mohl (6) remarked, »The very first observations showed me . . . that stomata under similar external conditions could behave very differently . . . for example, the stomata on the leaves of one plant regularly opened under water, whereas those of another closed.»

Similarly we find no change, either in aperture or starch, in sodium acetate solution. In sodium acetate buffer at pH 4.66 (dark only) the stomata closed as before; there appeared to be a slight increase in starch, but we could not be certain of this. In potassium acetate buffer at pH 4.60 (dark only) a similar closure occurred and the starch increase was more convincing, though still not indisputable.

IV. Conclusions

We consider that the foregoing few results justify the following statements:

1. The stomata open when epidermal strips are immersed in neutral, unbuffered salt solutions. Unless due account is taken of such »salt effects» in interpreting the results, similar experiments using buffer solutions provide no reliable evidence of the response of stomata to changes in pH. The closure in acetate buffer at c. pH 4.6 shows no more than that stomata will always close if the solution is made sufficiently acid — a fact well established by Arends for eight different acids, and later noted by Scarth (9) for acetic acid.

2. Such movements are in many cases light-sensitive, and this factor must be controlled in carrying out experiments of this type.

3. As with normal light-dark changes on the intact leaf, *Pelargonium* appears capable of appreciable stomatal movement without evident starch-change; we do not claim that such a change does not occur, but only that it is small compared with variations due to other causes, so that it cannot be convincingly demonstrated by simple inspection. In sodium acetate solution, however, *Pelargonium* does show a starch change of hitherto unrecorded type — rapid complete disappearance of starch from individual guard-cells.

We wish to express our gratitude to Mr. G. Butler, of this College, for the provision of the necessary *Pelargonium* plants; and to those members of the Chemistry and Physiology Departments who assisted us in matters relating to the preparation and properties of buffer solutions. We are indebted to Mr. G. S. Spencer for the accompanying photographs.

References

1. Alvim, P. de T.: Studies on the mechanism of stomatal behaviour. *Am. J. Bot.* 36: 781. 1949.
2. Arends, J.: Über den Einfluss chemischer Agenzien auf Stärkegehalt und osmotischen Wert der Spaltöffnungsschliesszellen. — *Planta* 1: 84. 1926.
3. Glasstone, S.: The electrochemistry of solutions. — London, 1930.
4. Heath, O. V. S.: Role of starch in light-induced stomatal movement, and a new reagent for staining stomatal starch. — *Nature* 159: 647. 1947.
5. — Studies in stomatal behaviour. II. The role of starch in the light response of stomata. Part 1. Review of literature, and experiments on the relation between aperture and starch content in the stomata of *Pelargonium zonale*. — *New Phytol.* 48: 186. 1949.
6. von Mohl, H.: Welche Ursachen bewirken die Erweiterung und Verengung der Spaltöffnungen? — *Bot. Zbl.* 14: 697 & 713. 1856.
7. Saïd, H. & Tolba, M. K.: The effect of pH and the kind of buffer system on the stomatal movements and starch-contents of the guard-cells. — *Bull. Fac. Sci. Fouad I Univ.* 27: 41. 1948.
8. Sayre, J. D.: Physiology of stomata of *Rumex patientia*. — *Ohio J. Sci.* 26: 233. 1926.
9. Scarth, G. W.: The influence of H-ion concentration on the turgor and movement

- of plant cells with special reference to stomatal behaviour. — Proc. Int. Cong. Pl. Sci., Ithaca, p. 1151. 1929.
10. Small, J. & Maxwell, K. M.: pH phenomena in relation to stomatal opening. I. *Coffea arabica* and other species. — *Protoplasma* 32:272. 1939.
 11. Small, J., Clarke, M. I., & Crosbie-Baird, J.: pH phenomena in relation to stomatal opening. II—V. — *Proc. Roy. Soc. Edin. B.* 61:233. 1942.
 12. Williams, W. T.: Studies in stomatal behaviour. IV. The water-relations of the epidermis. — *J. Exp. Bot.* 1:114. 1950.
 13. — & Spencer, G. S.: Quantitative estimation of stomatal starch. — *Nature* 166:34. 1950.

The Path of Carbon in Photosynthesis, XI. The Role of Glycolic Acid

By

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The participation of glycolic acid in plant metabolism has been considered in the past, but its relationship to the intermediates of carbon dioxide reduction has been obscure.

Kolesnikov (10) reported accumulation of glyoxylic acid in *Chlorella* during illumination. Anderson (1), Kolesnikov (11) and Clagett, Tolbert and Burris (9, 12) studied the non-photosynthetic metabolism of glycolic acid by plant tissues. The presence of glycolic acid among the early photosynthetic intermediates has been observed by Benson and Calvin (4) and by Burris, Wilson and Stutz (6).

The first stable product of carbon dioxide assimilation in photosynthesis had been shown to be carboxyl-labeled phosphoglyceric acid (Benson, et al., 5 and Calvin, et al., 7). Experiments were then designed to force the accumulation of the C₂ precursor of the α and β carbon atoms of this compound by illuminating plants in the absence of carbon dioxide (Benson and Calvin, 4). These conditions resulted in the accumulation of large amounts of glycolic acid and glycine.

The immediate precursor of the carboxyl labeled phosphoglyceric acid is of the reduction level of acetaldehyde or glycolaldehyde. Consequently, the accumulated glycolic acid appears to be either a precursor of the more reduced C₂ compound or a product of its oxidation.

It has been shown (Calvin, et al., 8) that glycolic acid appears subsequent to carboxyl-labeled compounds in photosynthesis and is not observed in very short periods of photosynthesis (1 Sec. Photosynthesis by Barley). Since

glycolic acid accumulates in the absence of carbon dioxide, it cannot arise from the condensation of two C_1 compounds directly formed from CO_2 but rather is probably derived from larger molecules. Since the compounds identified as phosphate esters in radiograms of $C^{14}O_2$ photosynthesis experiments have been found to contain derivatives of glycolic acid (to be published), it is possible that glycolic acid reacts metabolically in a phosphorylated form.

The close relationship of glycolic acid to the α and β carbon atoms of glyceric acid was first observed by Bassham, et al. (2, 8). Even in very short photosyntheses with $C^{14}O_2$ (4 Sec. Photosynthesis by Barley) the carbon atoms of glycolic acid were uniformly labeled. This acid has been degraded by lead tetraacetate oxidation and the products isolated as barium carbonate and formaldehyde-2,4-dinitrophenylhydrazone from the carboxyl and α carbon respectively. The carboxyl and α carbon atoms of glycolic acid and the α and β carbon atoms of phosphoglyceric acid are invariably uniformly labeled. This relationship has now been tested by a study of the conversion of synthetic radiomeric glycolic acids to phosphoglyceric acid during photosynthesis by *Scenedesmus*.

Experimental Procedure

Labeled glycolic acids prepared by Dr. B. M. Tolbert of this laboratory were added in the dark, during anaerobic illumination and during photosynthesis in $C^{12}O_2$ by *Scenedesmus*. Since the absorption of a strong acid such as glycolic acid is generally slow at physiological pH values, the experiments were performed in dilute phosphate buffer at pH 2.8. It has been observed in this laboratory (C. Ouellet — To be published) that photosynthetic ability at low pH (1.5 or less) is neither readily nor irreversibly destroyed and that the products are apparently normal.

Feeding Experiments. — A suspension of one gram of a two day old culture of *Scenedesmus* (Strain D_3 , Gaffron) in a 1 cm thick glass vessel (Benson, et al., 3) containing 50 ml of .001 M phosphate buffer, pH 2.8 was allowed to photosynthesize in air for 30 minutes with a light intensity of 2000 foot candles from both sides. A temperature of $20^\circ C$ was maintained by use of adequate infrared absorbers. The cells were allowed a twenty-minute adaptation period in the gas used immediately previous to adding the radioactive glycolic acid. In the dark experiments, the vessel containing the cell suspension was covered with black cloth during the experiment and the funnel was shielded from light during filtration and killing.

Labeled calcium glycolate-2 C^{14} (4.0 $\mu c/mg$) was decationized with Dowex-50. The free acid (3.2 mg, i.e. 0.0008 M) was added at zero time and the photosynthesis continued for ten minutes. The cells were then filtered rapidly from the solution in the light and killed by pouring hot absolute ethanol on the filter. By use of filter aids on a funnel equipped with two receivers and a two-way stopcock the killing procedure required 2–5 seconds. The algae were re-extracted with 50 % ethanol and hot water. The extracts were concentrated and separated by two-dimensional paper chromatography (Benson, et al., 5). After extraction, the insoluble materials

Table 1. *Products of Glycolic Acid Assimilation by Scenedesmus.*

	Light Assimilation 10 Min.				Dark Assimilation 10 Min.			6 Hour Dark Assimilation N ₂ + 1/2 0/0 CO ₂
	N ₂	Air	Air ¹	N ₂ + 1/2 0/0 CO ₂	N ₂	Air	N ₂ + 1/2 0/0 CO ₂	
C ¹⁴ in Lipids	120 (14 0/0)	76 (9 0/0)	39 (8 0/0)	48 (11 0/0)	3 (2 0/0)	15	1 (1 0/0)	10
C ¹⁴ in Insoluble Protein and Carbo- hydrates	400 (46 0/0)	270 (34 0/0)	110 (24 0/0)	280 (63 0/0)	27 (21 0/0)		30 (27 0/0)	
C ¹⁴ in Water Soluble Products	350 (40 0/0)	460 (57 0/0)	315 (68 0/0)	115 (26 0/0)	100 (77 0/0)	310	81 (72 0/0)	210
Total Glycolic Acid fixed ² (mg)	0.66	0.61	0.25	0.34	0.099		0.085	

C¹⁴ data are expressed in thousands of counts (9 dis./count) per minute.

¹ Glycolic acid-1-C¹⁴ Assimilation. All others are Glycolic acid-2-C¹⁴ experiments.

² Calculated from C¹⁴ found in assimilation products and the specific activity of the substrate.

(protein and polysaccharide) were counted directly and as barium carbonate after combustion. The lipid materials, separated from other soluble compounds in the chromatograms, were eluted and counted directly. The total glycolic acid fixed is then the sum of the three fractions and is tabulated in Table 1. From the known specific activity of the glycolic acid the amount assimilated during the experiments was calculated and is tabulated in Table 1. The products were observed on radiograms of such chromatograms and were identified where necessary by cochromatography with authentic materials. The amount of C¹⁴ in each of a number of the major products was counted directly on the paper and is tabulated in Table 2. A typical pair of radiograms is shown in Figure 1.

Degradation of Phosphoglyceric acids. — The radioactive area corresponding to phosphoglyceric acid was eluted from the paper and hydrolyzed for 30 hours in 1.0 N hydrochloric acid. The total hydrolysate was chromatographed and the resultant glyceric acid spot was cocrystallized with 50 mg of authentic calcium glycerate. The fact that the resulting product possessed the calculated specific activity serves as added evidence of identity. Glyceric acid was degraded according to the method of Bassham, et. al. (2).

Degradation of Glycolic Acid. — A tracer quantity of C¹⁴-labeled glycolic acid, obtained by elution from a paper chromatogram was added to 30.4 mg of glycolic acid in three ml of glacial acetic acid in a small flask. The solution was frozen, about 0.5 g lead tetraacetate was added, and the flask was attached through a stopcock to an inverted U-tube. The system was evacuated, the stopcock closed and the reaction mixture refluxed on a water bath at 90° C for thirty minutes. After cooling, the volatile contents of the flask were distilled through the U-tube into a second flask containing 80 mg of 2,4-dinitrophenylhydrazine and immersed in liquid nitrogen.

The stopcock was again closed and the second flask warmed until a clear yellow solution was obtained. The first flask was replaced by a third flask containing

Table 2. *Water Soluble Products of Labeled Glycolic Acid Assimilation by Scenedesmus.*

Compound	Ten Minute Light Assimilation				Ten Minute Dark Assimilation			Six Hour Dark N ₂ +1/2 ^{0/0} CO ₂ ¹
	N ₂ ¹	Air ¹	Air ²	1/2 ^{0/0} CO ₂ +N ₂ ¹	N ₂ ¹	Air ¹	N ₂ +1/2 ^{0/0} CO ₂ ¹	
Phosphoglycerate	26	19	11	5.3	10	22	17	6
Hexose Phosphates ...	6.2	6	13	6.9	3	9.4	1.5	3
Triose Phosphates	3.3	2.5	2	2.9	4	2.8	4	1
Phosphopyruvate	6.2	3.9	3		3	6.1		3
Sucrose	2.2	9.5	12	16		2.0		3
Polysaccharides	5.7	7.5	13	18	2	1.5		
Serine	23	8	13	32	37	12	34	3.5
Glycine	1.4	1.5	2	2.9	25	1.7	31	2
Alanine	1.5	3.3	1.5	2.0	7	3.7	9	9
Aspartic	5.9	5.5	7.5	3.1		6.1		3
Glutamic	2.7	8.5	4.5	2.5	3	18	2	40
Glutamine	0	0.7	3		1	0.9		3
Glyceric	1.9	3	2	1.8	1	5.8	1	
Citric	1.6	4.5	3.5			2.1		2
Succinic	1.3	1.8	0		2	2.7		3
Unk. Spot under Lipids	4.1	10	2	3.6				15
Malic	7.0	8.5	7	3.5	2	3.3	1	8

¹ Glycolic acid-2-C¹⁴ assimilation.² Glycolic acid-1-C¹⁴ assimilation.

5.0 ml saturated, carbonate-free, sodium hydroxide solution. Both flasks were immersed in liquid nitrogen for a few minutes, the stopcock was opened and the system was evacuated. The liquid nitrogen bath was removed from the second flask and the volatile contents distilled into the third flask. The residue of formaldehyde-2,4 dinitrophenylhydrazine in the second flask was purified chromatographically on silicic acid and the specific activity determined. This specific activity, together with the theoretical yield gives the total activity of the alpha carbon atom. The third flask was warmed to room temperature, and the solution contained therein yielded, upon addition of barium chloride solution, a precipitate of barium

Table 3. *C¹⁴ Distribution in Glycolic Acid and Glyceric Acid.*

	4 Sec. PS Barley C ¹⁴ O ₂	10 Min. PS Scenedesmus, Air, C ¹⁴ H ₂ OH-COOH	10 Min. PS Scenedesmus, N ₂ , C ¹⁴ H ₂ OH-COOH	10 Min. PS Scenedesmus, Air CH ₂ OH-C ¹⁴ OOH
Glycolic				
CH ₂ OH	51	100	100	0
COOH	49	0	0	100
Glyceric				
CH ₂ OH	6.8	49	48	29
CHOH	6.5	—	56	24
COOH	87	8.6	7.2	46

Percentages given in terms of measured starting activity.

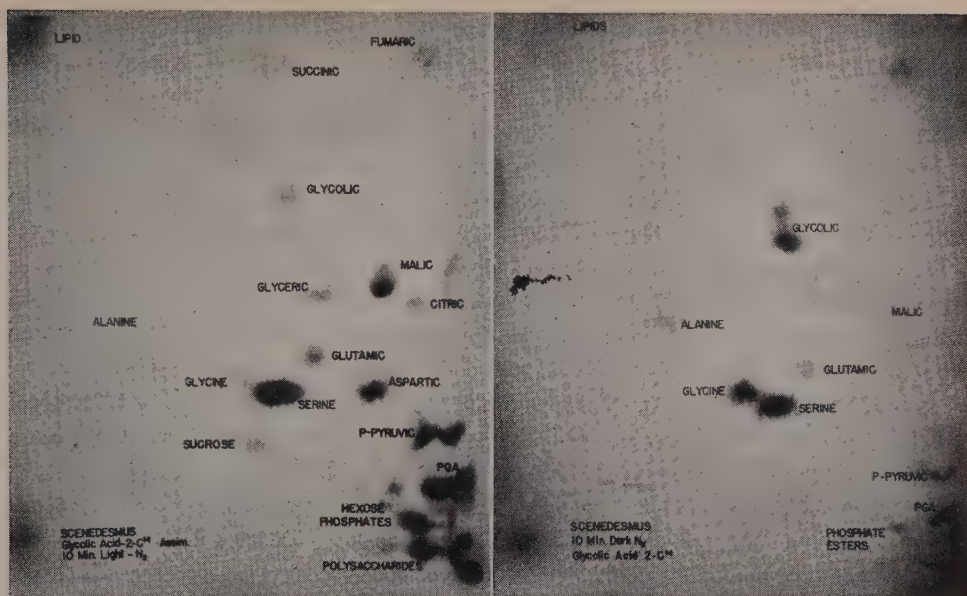


Fig. 1. C^{14} radiograms of ten minute dark and light glycolic acid-2- C^{14} fixation by *Scenedesmus* in nitrogen. Solvents used in developing the chromatograms were phenol and butanol-propionic acid-water solution (Benson, et al., 5).

carbonate which was washed, dried, weighed and counted. The product of the specific activity of the barium carbonate and the total yield (slightly greater than theoretical due to introduction of inactive carbon dioxide in reagents and manipulation) gives the total activity of the carboxyl carbon of glycolic acid. These results are tabulated in Table 3.

Results and Discussion

Dark Metabolism of Glycolic Acid

It has been shown (Benson, et al., 4) that glycolic acid is readily metabolized in the dark by barley leaves and algae. In the experiments described below there is evidence that the assimilation of glycolic acid proceeds by paths other than intermediate conversion to carbon dioxide.

Short Dark Experiments. — The major products of six to ten minute dark anaerobic assimilation of glycolic acid are glycine and serine, Figure 1. The conversion of labeled glycolic acid to glycine in barley sap has also been observed in this laboratory. The appearance of labeled serine suggests that a reaction similar or identical with the Sakami reaction may occur in plant

tissue (this reaction has also been observed in this laboratory in *Scenedesmus* which have been fed 1-C^{14} or 2-C^{14} labeled glycine). Conversion to serine is appreciably faster in the light than anaerobically in the dark where both amino acids appear in like amounts and the ratio of serine to glycine varied from unity to 1.5 in all experiments. No relationship between the nature of the anaerobic flushing gas mixtures and the serine-glycine ratio was observed. In air the ratio of serine to glycine increased to 7. An appreciable synthesis of sucrose, phosphoglyceric acid and polysaccharide occurred in ten minute dark aerobic assimilation. Apparently, energy is derived from oxidative processes and is used for carbohydrate synthesis. Fat synthesis corresponding to about 5 % of the soluble products was observed.

In all dark assimilation experiments considerable amounts of unchanged glycolic acid is found in the cell extract. In the light, little excess glycolic was observed in the similarly prepared extracts.

Long Dark Experiments. — The further metabolic products of glycine, serine and glycolic acid were observed in radiograms of 30 minute to 6 hour dark assimilation experiments with glycolic acid- 2-C^{14} . The soluble products formed during 6 hours dark assimilation of glycolic acid- 2-C^{14} are given in Table 2. It is apparent from the large fraction of glutamic, succinic, fumaric, malic and citric acids that considerable oxidation through the tricarboxylic acid cycle may have occurred. This metabolic course appears similar to that observed in unpublished experiments with *Scenedesmus* which have been fed 2-C^{14} glycine in the dark for similar periods. Since flushing with inert carbon dioxide did not affect the results, exchange of C^{14}O_2 arising from oxidation of the substrate into the tricarboxylic acid cycle is not likely. The possibility should be pointed out that a C_2 compound related to glycolic acid (glycine) may be condensed through reversible reactions to a C_4 compound and enter the tricarboxylic acid cycle as oxalacetate. Otherwise, it must be further reduced to react as acetate in this cycle.

Light Assimilation Experiments

The products of ten minute and longer periods of illumination were studied and found generally similar to the products of photosynthesis with C^{14}O_2 . Glycolic acid is converted to cell material (protein, polysaccharides) and fats. The radioactivity in the various reservoirs of soluble products corresponded to that observed when C^{14}O_2 is assimilated at this high pH.

The conversion of glycolic acid to serine was observed in the light as well as in the dark. In all cases the ratio of serine to free glycine was very high.

The radioactivity in glutamic acid, which may be taken as a measure of

respiration of labeled intermediates via the tricarboxylic acid cycle, was greater in the aerobic experiments. A similar result was observed in the aerobic dark experiment.

When the algae were flushed with $C^{12}O_2$ prior to and during the glycolic acid assimilation, serine, sucrose and polysaccharide (containing glucose) reservoirs accumulated the largest fraction of radioactivity. At the same time, radioactivity in phosphate esters was greatly diminished. This can be attributed to the dilution of C^{14} in the intermediates by the $C^{12}O_2$. The sucrose reservoir, which is much larger, would rapidly acquire a greater total radioactivity, although the specific activity would be low.

In experiments without added carbon dioxide, photosynthesis would be expected to occur at a slower rate. In air the natural CO_2 concentration should be sufficient to allow a moderate accumulation of activity in the sucrose reservoir, whereas in nitrogen without added carbon dioxide, a much smaller amount of sucrose would be formed. Radioactivity in the phosphate compounds, intermediate in sucrose synthesis, should be inversely affected. With diminishing dilution by added carbon dioxide the specific activity of these small reservoirs would be increased and their relative radioactivities probably would represent a measure of dilution of assimilated glycolic acid by carbon dioxide.

In the low carbon dioxide pressure experiments (air and nitrogen) an amount of labeled phosphoglyceric acid, large compared to that of normal photosynthesis was observed. The significance of this observation may well lie in the effect of pH or CO_2 concentration upon the reservoir sizes. The phosphoglyceric acid of the aerobic experiment was degraded and the results are tabulated in Table 3.

Carboxyl-Labeled Glycolic Acid Assimilation. — An identical experiment was performed in air with glycolic acid-1- C^{14} . The distribution of C^{14} in the products, Table 2, was similar to that observed in aerobic glycolic acid-2- C^{14} assimilation. The phosphoglyceric acid obtained in this experiment was degraded and the results are tabulated in Table 3.

Degradation Results. — The accumulation of C^{14} in nearly equal amounts in the α and β carbon atoms of phosphoglyceric acid during photosynthesis has now been observed when glycolic acid is the labeled substrate. In Table 3 it is seen that radioactivity of α and β carbon atoms in both cases are approximately equal. It is possible that the equal α and β labeling in C_3 compounds may arise in a number of ways. However, one possibility, consistent with our previous observations (Calvin, et al., 8), would be that some symmetrical intermediate or compound existing in rapid equilibrium with an intermediate lies between glycolic acid and the C_2 carbon dioxide acceptor molecule.

Summary

The metabolism of C^{14} labeled glycolic acid by *Scenedesmus* has been studied using radiochromatographic techniques for the separation and identification of products.

When the pH of the medium was 2.8, appreciable assimilation occurred. The products were identical to those observed in $C^{14}O_2$ photosynthesis.

A major reaction anaerobically in the dark resulted in incorporation of C^{14} in almost equal amounts in the glycine and serine reservoirs. When the algae were illuminated, the glycine and glycolic acid radioactivity decreased.

Aerobic and anaerobic glycolic acid assimilation was studied during photosynthesis. The C^{14} level in the sucrose and the intermediates of its synthesis varied with $C^{12}O_2$ pressure in the gas used.

1- C^{14} and 2- C^{14} glycolic acids give similar distribution of radioactivity in the products.

Phosphoglyceric acid isolated from the products of assimilation of both glycolic acids was degraded and found to be approximately equally labeled in its α and β carbon atoms.

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References

1. Anderson, E. H.: Studies on the Metabolism of the Colorless Alga *Prototheca zopfii*. — *J. Gen. Physiol.* 28: 297. 1945.
2. Bassham, J. A., Benson, A. A. and Calvin, M.: The Path of Carbon in Photosynthesis VIII. The Rôle of Malic Acid. — *J. Biol. Chem.* 185: 781. 1950.
3. Benson, A. A., Calvin, M., Haas, V. A., Aronoff, S., Hall, A. G., Bassham, J. A. and Weigl, J. W.: C^{14} in Photosynthesis. — *Photosynthesis in Plants*, Iowa State College Press, Ames, Iowa, Chap. 19: 381. 1949.
4. Benson, A. A. and Calvin, M.: The Path of Carbon in Photosynthesis VII. Respiration and Photosynthesis. — *J. Exptl. Bot.* 1: 63. 1950.
5. Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. and Stepka, W.: The Path of Carbon in Photosynthesis V. Paper Chromatography and Radioautography of the Products. — *J. Am. Chem. Soc.* 72: 1710. 1950.
6. Burris, R. H., Wilson, P. W. and Stutz, R. E.: Incorporation of Isotopic Carbon into Compounds by Biosynthesis. — *Botan. Gaz.* 111: 63. 1949.
7. Calvin, M. and Benson, A. A.: The Path of Carbon in Photosynthesis. — *Science* 107: 476. 1948.
8. Calvin, M., Bassham, J. A., Benson, A. A., Lynch, V., Ouellet, C., Schou, L., Stepka, W. and Tolbert, N. E.: The Path of Carbon in Photosynthesis X. Carbon Dioxide Assimilation in Plants. — *Proc. Soc. Exptl. Biol.* (in press).
9. Claggett, C. O., Tolbert, N. E. and Burris, R. H.: Oxidation of α -Hydroxy Acids by Enzymes from Plants. — *J. Biol. Chem.* 178: 977. 1949.

10. Kolesnikov, P. A.: Die Rolle der organischen Säuren im Kohlehydratstoffwechsel bei einem einzelligen grünen Organismus (Chlorella). — Doklady Akad. Nauk. 27: 353. 1940.
11. — The Rôle of Glycolic Acid in the Oxidation Metabolism of Green Cells. — Biochemia 13: 370. 1948.
12. Tolbert, N. E., Clagett, C. O. and Burris, R. H.: Products of the Oxidation of Glycolic Acid and L-Lactic Acid by Enzymes from Tobacco Leaves. — J. Biol. Chem. 181: 905. 1949.

The Ion Absorption in Roots Lacking Epidermis

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It has been shown in two previous communications (Burstöm, 1 and 2) that di-*n*-amylacetic acid (DAA) has specific histological effects on roots. The acid peels off the epidermis of roots without affecting the cortical parenchyma. It does not interfere in the auxin mechanism. It has also been shown that, although the rate of the nitrate assimilation decreases in DAA-treated roots, there is a significant increase in the amount of stored nitrate. These findings gave rise to speculations regarding the localization of the ion absorption. The following experiments have been carried out in order to study the ion absorption in roots lacking epidermis.

Material and Methods

The test material has been plants of Weibull's Eroica wheat. The plants were grown in well-aerated nutrient solutions at 20° C under artificial illumination. The solution had the following composition: KH_2PO_4 1/1000, $\text{Ca}(\text{NO}_3)_2$ 1/1000, MgSO_4 1/2000, $\text{FeC}_6\text{H}_5\text{O}_7$ 1/50000 and MnSO_4 1/50000. All concentrations are in mol per litre. The plants thus had only half the normal concentrations of potassium and calcium and no chloride at all, in order to insure a greater absorption of these ions from the test solutions. The nutrient solutions were renewed daily or every second day. The pH in fresh solutions amounted to 5.3. In old solutions without DAA the pH amounted to 6.3 and with DAA to 6.0. This is of great importance to the dissociation of DAA, which in the different series may be regarded as constant. (Concerning the dissociation of DAA see Burstöm, 1.) Each of about

Table 1. *The test solutions and the pH of the four treatments which constitute one series of experiments.*

Treatment	Volume	Test solutions in mol	Initial pH	Final pH
A	100 ml.	KCl 1/200	5.3	6.1
B	100 ml.	DAA + KCl 1/200	5.4	5.8
C	100 ml.	CaCl ₂ 1/400	5.2	6.1
D	100 ml.	DAA + CaCl ₂ 1/400	5.1	5.9

fifteen series of experiments consisted of four different treatments, each with four duplicates (one duplicate=14 plants). When the plants had grown for one day (in series 2 four days) in the nutrient solution, half the series were placed in nutrient solutions with DAA added in the following molar concentrations: Series 1: 10^{-5} , series 2 and 3: $2 \cdot 10^{-5}$, and series 4 and 5: $3 \cdot 10^{-5}$. Series 4 is a repetition of the experiments of series 5. The absorption test began when the plants were eleven days old and went on for three days. Before the test the plants were cleaned and the seeds removed. The histological effect of DAA was also microscopically controlled. The test solutions were as shown in table 1. The acid had the same concentration as in the nutrient solution and was regarded as absorbed after one day. For this reason DAA was added again on the second and the third days. Even phosphate was added every day in order to reduce the increase in pH. Without phosphate the pH would increase to about 7 and the histological effects would not appear in the outgrowing parts of the roots.

By weighing before and after the test the solution volumes, the water absorption, the transpiration, the fresh and the dry weights of the plants were measured. The percentage of water in the plants was also computed. The concentrations of chloride before and after the test were measured by potentiometrical titration with 0.010 AgNO₃. The phosphate concentrations were measured colorimetrically according to Scheel (Z. anal. Ch., 105, 1936). The cation excess was computed afterwards by titration with 0.107 N KOH. The original phosphate concentration was impossible to measure for technical reasons. The figures in table 4, which represent the phosphate concentrations are only theoretical values.

Experiments were also carried out with L-malic acid, DL-malic acid or acetic acid instead of DAA in order to see whether the histological effects were specific for DAA or not.

During the test no consideration was taken of the evaporation from the surface of the solution. This evaporation amounted to about 4 g. per duplicate.

The values in table 2, which is an example of the method, belong to series 3.

Table 2. *An example of the method taken from series 3.* All values except those of the dry weight represent the average of four duplicates. The dry weight values represent the total of four duplicates, i.e., one treatment.

Series	Fresh weight g.		Dry weight g. 4/1	
	Leaves	Roots	Leaves	Roots
KCl	3.91	1.95	1.88	0.56
DAA + KCl	2.41	1.52	1.40	0.58
CaCl ₂	3.77	1.95	1.83	0.59
DAA + CaCl ₂	2.24	1.35	1.32	0.55

Series	Water uptake g.	Transpiration g.	Water in per cent of fresh weight	
			Leaves	Roots
KCl	45.56	45.48	87.98	92.82
DAA + KCl	36.14	36.02	86.51	90.46
CaCl ₂	54.40	53.97	87.86	92.42
DAA + CaCl ₂	30.77	30.87	85.27	89.81

Series	Milliequivalents					
	Chloride			Phosphate uptake	Cation	
	Initial	Final	Uptake		Excess	Uptake
KCl	0.519	0.369	0.150	0.080	0.043	0.187
DAA + KCl ...	0.541	0.364	0.177	0.063	0.026	0.214
CaCl ₂	0.510	0.306	0.204	0.083	0.043	0.244
DAA + CaCl ₂	0.510	0.353	0.157	0.057	0.009	0.205

Series	Absorption per g. living roots			
	m-equiv.			Water g.
	Chloride	Phosphate	Cations	
KCl	0.077	0.041	0.096	23.36
DAA + KCl	0.116	0.041	0.140	23.78
CaCl ₂	0.105	0.042	0.125	27.90
DAA + CaCl ₂	0.116	0.042	0.152	22.79

Results

The morphological effects of DAA were in accord with those described by Burström (1). By control before and after the DAA-treatment series 1 with the weakest DAA-concentration showed no root hairs and no rupturing epidermis. Series 2—5 showed a typically rupturing epidermis. In series 2 those rapidly ruptured cells were found only on the younger parts of the roots. In all other experiments they were found all along the roots. Those roots were regarded as having no functioning epidermis. The figures 1 A—C are taken from series 3. Figure 1 A shows an eleven day old control root

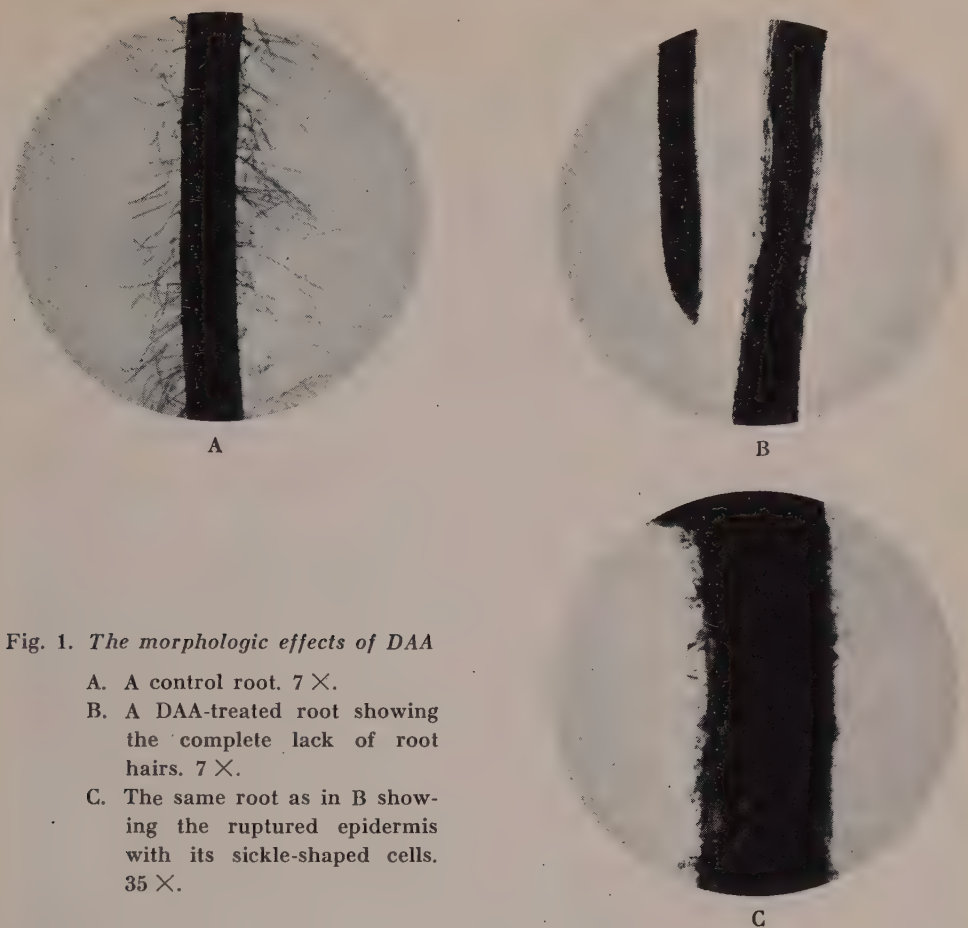


Fig. 1. *The morphologic effects of DAA*

- A. A control root. 7 \times .
- B. A DAA-treated root showing the complete lack of root hairs. 7 \times .
- C. The same root as in B showing the ruptured epidermis with its sickle-shaped cells. 35 \times .

which has remained in a solution of $1/400$ -mol CaCl_2 for three days. Figure 1 B is a root from the parallel treatment i.e., after three days in a solution of $1/400$ -mol CaCl_2 and $2 \cdot 10^{-5}$ -mol DAA. This root is completely lacking in root hairs. Figure 1 C is the same root in higher magnification showing the ruptured epidermis. Figure 2 is an intercellularly observed in the third pair of the adventitious roots from series 5. This shows that, although they were less susceptible, the epidermis of these roots was affected by DAA. It could not function as an intact epidermis. For this reason the adventitious roots were not removed before the plants were placed in the test solutions. The blackened part in the figure is dead cell substance not adhering to the remaining epidermis.

Roots which after a nine day treatment with $3 \cdot 10^{-5}$ DAA were placed in a common nutrient solution turned out to be as vital as the controls.

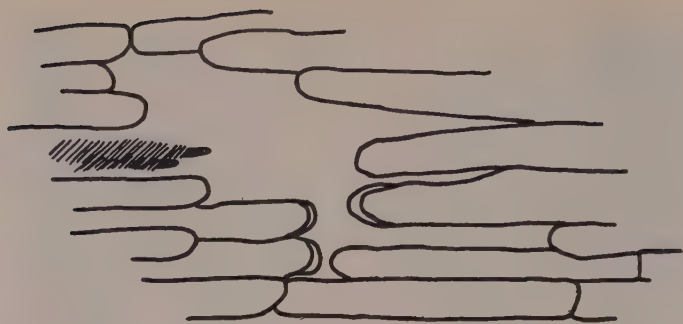


Fig. 2. *Intercellular of epidermis from an adventitious DAA-treated root.*

They continued to grow and the outgrowing parts gradually resumed their normal appearance.

The ion absorption in these roots lacking epidermis had in every experiment quite other proportions than in the unaffected control roots. All the values for the chloride, the phosphate, and the cation absorption were calculated in relation to the absorption of water. This was assumed to be independent of and unaffected by DAA. The values for the water absorption (see table 3) did not vary more than the values for the evaporation from the surface of the solution, and the values for the water content of the roots did not vary more than 2 per cent. For these reasons the water absorption was assumed to be constant.

The ion figures in table 4 represent m-equiv. per 100 ml. solution and per gram of living roots. The figures which best show the variations and similarities are the chloride concentration in the external solution before and after the test and the absorption of chloride during the test.

If we compare the absorption in normal roots with the absorption in acid-treated ones we find that the latter is considerable higher than that in the normal roots. This is true of anions and even more so of cations. The anion concentrations increased in series 1 (see table 4) from 0.490 to 0.794 and from 0.543 to 0.724 i.e., they increased by 0.304 and 0.181 m-equiv. The cation concentrations in the same series showed an increase of 0.432 and 0.242 m-equiv. As a rule the anion concentration increased about 25 per cent and the cation concentration about 50 per cent.

In normal roots there was an excess of anion absorption. The values in series 1 are 0.490 m-equiv. of anions against 0.325 m-equiv. of cations in KCl-solutions and 0.543 against 0.367 in CaCl_2 -solutions. *In roots lacking epidermis this excess tended to disappear.* The excess in the normal roots of

Table 3. *Water absorption during the tests. Cf. table 4.*

Treatment	Series				
	1	2	3	4	5
KCl	19.55	23.31	23.36	25.21	15.91
DAA + KCl	18.11	25.44	23.78	23.15	14.27
CaCl ₂	20.92	21.70	27.90	26.13	17.56
DAA + CaCl ₂	20.86	24.22	22.79	23.64	14.54

series 1 amounted to 0.165 and 0.176 m-equiv. and in roots without epidermis only to 0.127 and 0.115 m-equiv. These phenomena were the same in all series.

Another fact is that in intact roots the absorption of water was greater than the absorption of ions. In table 4 series 1 the final chloride concentrations in the external solution amounted to 0.582 and 0.569 m-equiv. The ratio between chloride and water absorbed amounted to 0.300 and 0.362 m-equiv. per 100 ml. As mentioned before the water absorption itself was the same whether the roots had their epidermis or not. *In roots without epidermis the rates of the absorption of ions and water were almost equal.* The external concentrations were about 0.51 m-equiv. and the absorbed amounts corresponded to 0.467—0.594 m-equiv. The differences in the absorption of potassium and calcium did not indicate anything of special interest in this investigation.

There is another observation which ought to be mentioned. The treatments in series 4 and 5 were equal but still there was a great difference in the results. In series 5 the concentration of ions in the roots and even in the control roots was much higher. This fact did not depend on a greater absorption of ions but on a smaller absorption of water. Series 4 had the same water absorption as the other series of experiments. I have no explanation of this phenomenon.

The experiments with L-malic acid, DL-malic acid, and acetic acid showed that these acids have no histologic effects. The epidermis of the acid-treated roots was quite similar to that of the intact ones. There were neither loosening of cells nor even formation of intercellularies. The ion absorption did not differ in any way from that of the control roots. In a treatment with DL-malic acid the chloride absorption per gram of living roots for KCl alone amounted to 0.139, for KCl and DL-malic acid to 0.140, for CaCl₂ to 0.070, and for CaCl₂ and DL-malic acid to 0.071 m-equiv. Thus we may assume that the effects of DAA are not characteristic of acids in general but are specific for this acid, and that *it is not the DAA itself but its histologic effects which cause the altered ion absorption.*

Table 4. *The influence of DAA on the ion absorption.* The DAA-treatments are as follows: Series 1: 10^{-5} mol DAA for 6 days, series 2: $2 \cdot 10^{-5}$ mol DAA for 3 days, series 3: $2 \cdot 10^{-5}$ mol DAA for 6 days, and series 4 and 5: $3 \cdot 10^{-5}$ mol DAA for 6 days. Roots treated with DAA are regarded as having no epidermis. The values are m-equiv. per 100 ml. solution.

Series	In the external solutions			Absorption			
	Chloride		Phosphate initial ¹	Cl/H ₂ O	P/H ₂ O	Anions/H ₂ O	Cations/H ₂ O
	Initial	Final					
1							
KCl	0.511	0.582	0.2	0.300	0.19	0.49	0.33
DAA + KCl	0.511	0.490	0.2	0.594	0.20	0.79	0.67
CaCl ₂	0.510	0.569	0.2	0.362	0.18	0.54	0.37
DAA + CaCl ₂	0.509	0.490	0.2	0.561	0.16	0.72	0.61
2							
KCl	0.529	0.621	0.3	0.330	0.18	0.51	0.39
DAA + KCl	0.530	0.550	0.3	0.492	0.20	0.69	0.58
CaCl ₂	0.519	0.609	0.3	0.377	0.16	0.54	0.35
DAA + CaCl ₂	0.510	0.530	0.3	0.467	0.21	0.68	0.60
3							
KCl	0.519	0.678	0.4	0.335	0.18	0.51	0.42
DAA + KCl	0.541	0.570	0.4	0.483	0.17	0.65	0.58
CaCl ₂	0.510	0.670	0.4	0.375	0.15	0.53	0.48
DAA + CaCl ₂	0.510	0.510	0.4	0.504	0.18	0.69	0.70
4							
KCl	0.299	0.300	0.4	0.300	0.36	0.66	0.53
DAA + KCl	0.519	0.511	0.4	0.552	0.27	0.82	0.75
CaCl ₂	0.269	0.241	0.4	0.292	0.36	0.65	0.47
DAA + CaCl ₂	0.510	0.490	0.4	0.558	0.27	0.83	0.68
5							
KCl	0.529	0.500	0.4	0.613	0.27	0.88	0.83
DAA + KCl	0.530	0.501	0.4	0.793	0.27	1.06	1.31
CaCl ₂	0.510	0.479	0.4	0.572	0.16	0.73	0.71
DAA + CaCl ₂	0.511	0.490	0.4	0.667	0.12	0.79	0.91

¹ Theoretical figures. Phosphorus solutions added in portions of one or two ml. each day; the variation may be as for »initial chloride».

Comments

A general review of the mechanism of the ion absorption may simplify the following attempt to explain the results reported here. Figure 3 (received from Burström) outlines the mechanism of the ion absorption based on the theories proposed by Lundegårdh (3) and Robertson (5). The absorption may be in a single cell or, as in this investigation, in a whole root. The outer level (o-level) represents the oxidizing phase where the absorption of ions from the external solution is localized. The inner level (i-level) represents

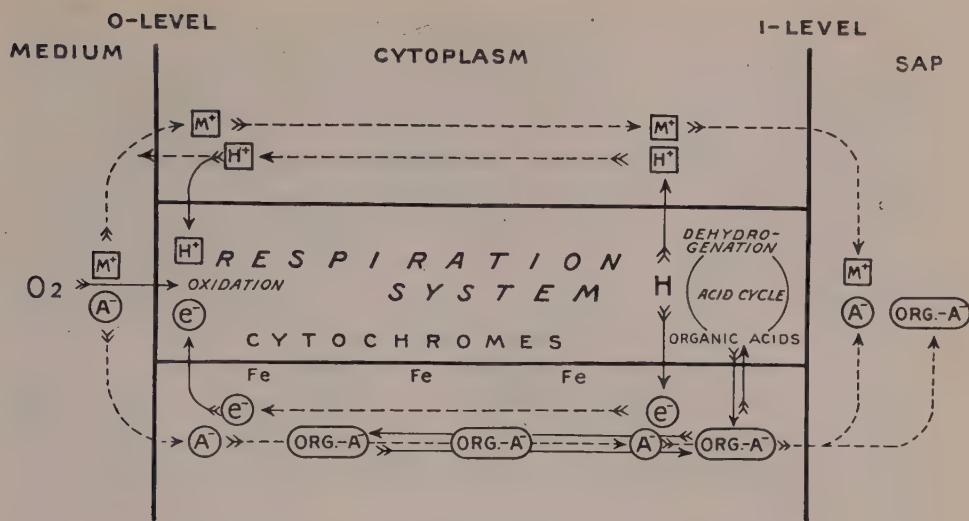


Fig. 3. Scheme of the mechanism of the ion absorption.

the reducing phase where the ions are exuded in the sap of the vessels (or of a vacuole). These two phases are different processes. The absorptions of cations and anions on the outer level are independent of each other. The cytoplasm has an acid character and contains acid groups dissociating hydrogen ions. These hydrogen ions can be exchanged for cations from the inner level and delivered to the sap.

The absorption of anions is connected with respiration, catalyzed by cytochrome systems. A ferrous ion is oxidized by giving off an electron and thus becomes trivalent. This ferric ion is able to absorb an anion from the external solution. The absorbed anion is then transported to the inner level. The free electron is used by the respiratory system. Together with atmospheric oxygen and hydrogen ions it forms water. The ferrous ion obtains its electron from the dehydrogenation system which should be located on the inner level. This system produces hydrogen which is capable of giving rise to electrons and hydrogen ions. If the accumulation of ions is unequal it is equilibrated either by the hydrogen ions if anions are in excess, or by organic ions if — as usually is the case — cations are in excess. The organic ions are supposed to be derived from the acid cycles of respiration. An acid cycle is a part of the dehydrogenation system. The dissociation of the acid and the cation excess balance each other. — This scheme outlines facts and assumptions of the ion absorption of intact normal roots.

In this investigation the ion absorption in roots not treated with DAA should be of this type. Roots treated as above with DAA have no epidermis. If the epidermis does not take part in the oxidizing phase the absorption in those roots should be the same as in the control ones. But it is a fact that the roots lacking epidermis show a significant increase in the ion absorption. Consequently some part of the epidermis may be concerned in the mechanism of the ion absorption. If the oxidizing phase is located in the epidermis and if there is no longer a selective valve there, the external solution streams to the inner level. The mechanism exuding salts and water at this level must still operate, but is now supplied with a solution of the composition of the external one, or very near so. The concentration in the sap will usually be the same. This makes the impression of a wholly passive uptake of salts in proportion to the water absorption, which means that the whole active mechanism should be located to the epidermis. However, in some instances the salt absorption decidedly exceeded that of water. This surplus may indicate an active system on the inner level, which takes care of the ions in the concentrations in which they are delivered from the outer parts but pumps the ions to the sap without any selective action. Lundegårdh (4) questions in a recent paper the occurrence of a metabolic exudation at the inner level. If our interpretation is correct, it implies that there may be an actively accumulating mechanism at work, even if the selective mechanism, apparently located in the epidermis and identical with the oxidizing system, is out of function.

In this way the relation can be explained between the absorption of ions and water in roots lacking epidermis. Thus it is possible to explain the results of this investigation along the lines of the general theories of ion absorption. The investigation may show that a selective absorption of ions takes place in the epidermis and that the oxidizing phase of ion absorption is situated there.

Summary

The ion absorption of wheat roots with and without epidermis has been studied. The epidermis was shed off the roots by treatment of di-*n*-amyl-acetic acid.

In roots lacking epidermis the absorption of anions and of cations increased. The ratio between absorbed amounts of ions and water approached that of the external solution. The water absorption was assumed to be constant. The external solution may stream to the inner level, so that salts and water are exuded in the sap in concentrations more equal to that of the external solution. That means that the oxidizing phase with the selective absorption of ions should be located in epidermis.

References

1. Burström, H.: Studies on growth and metabolism of roots. I. The action of *n*-diamylacetic acid on root elongation. — *Physiol. Plant.* 2:197. 1949.
2. — Studies on growth and metabolism of roots. II. *N*-diamylacetic acid and assimilation of nitrate. — *Ibid.* 2:332. 1949.
3. Lundegårdh, H.: Absorption, transport and exudation of inorganic ions by the roots. — *Arkiv f. Bot.* 32 A, Nr. 12. 1945.
4. — The translocation of salts and water through wheat roots. — *Physiol. Plant.* 3:103. 1950.
5. Robertson, R. N. and Wilkins, M. J.: Studies in the metabolism of plant cells. — *Austr. J. Scient. Res.*, 1:17. 1948.

